MOUSE BRAIN GLUCOCORTICOID RECEPTORS

BY

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This dissertation is dedicated to the memory of

Clarence Phillips Connell

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Glucocorticoid binding sites in cytosol prepared from whole brains of female CD-1 mice perfused 3-5 days after ovariectomy-adrenalectomy were studied by equilibrium, kinetic and transport methods. In the standard buffer (containing 10 mM Na_2MoO_4 and 2 mM dithiothreitol, DTT) both unoccupied and occupied binding sites for [^{3}H]dexamethasone (DEX) were stable at 2°C. The absence of DTT resulted in rapid loss of unoccupied sites, and the absence of molybdate resulted in loss of unoccupied sites with a $t_{\frac{1}{2}}$ of 1 h at 12°C and 10 h at 2°C.

Equilibrium isotherms revealed one apparent class of saturable, high-affinity binding sites (each) for [^{3}H]DEX, [^{3}H]triamcinolone acetonide (TA), and [^{3}H]corticosterone (B), but the concentration of sites for [^{3}H]DEX and [^{3}H]TA (putative receptors) was only 63% of the complete ensemble of [^{3}H]B sites. The concentration of DEX-displaceable [^{3}H]B sites was equivalent to the receptor concentration measured with

[^{3}H]DEX and [^{3}H]TA. DEX failed to interact with 37% of the [^{3}H]B sites; these sites resembled corticosterone binding globulin (CBG). The (decreasing) order of steroid affinity for the putative receptors measured with the [^{3}H]ligands was: [^{3}H]TA > [^{3}H]DEX > [^{3}H]B. The competing steroids that were tested fell into the following order of decreasing affinity (increasing K_{di}) for the [^{3}H]dexamethasone binding sites: DEX > B > 11-deoxycorticosterone > progesterone $\simeq$ cortisol > aldosterone > cortexolone > testosterone.

Measured rate constants for the association of [^{3}H]DEX, [^{3}H]TA, and [^{3}H]B with the receptors were very similar. The very different affinities of these agonist ligands resulted from their quite different dissociation rate constants. Progesterone at concentrations greater than 10^{-6} M (but not DEX itself) significantly accelerated dissociation of the [^{3}H]DEX-receptor complexes.

Nonactivated [^{3}H]TA-receptor complexes possessed Stokes radius 77 Å, sedimentation coefficient 9.7 S, and molecular weight 315,000 daltons; heat-activated [^{3}H]TA-receptor complexes (confirmed by DNA-cellulose binding) possessed Stokes radius 58 Å, sedimentation coefficient 3.7 S, and molecular weight 90,000 daltons. Physical characteristics of the cytosol CBG-like binder (Stokes radius 46 Å, sedimentation coefficient 4.1 S, molecular weight 70,000 daltons) were indistinguishable from those of plasma CBG.

CHAPTER I

GENERAL INTRODUCTION

Biosynthesis, Secretion, and Metabolic Effects of Glucocorticoids

The adrenal cortex produces over forty steroids, but only a few of these are secreted in biologically significant quantities. The major active secretions are classified as glucocorticoids (e.g., cortisol, corticosterone, 11-deoxycortisol) or mineralocorticoids (e.g., aldosterone, deoxycorticosterone), with 21 carbon atoms, and weak androgens (e.g., dehydroepiandrosterone), with 19 carbon atoms (see Table 1-1). Minor amounts of progestational and estrogenic steriods are also secreted. The predominant glucocorticoid is cortisol in some species, e.g., hamster, sheep, and primates, but it is corticosterone in others, e.g., rat, mouse, and rabbit (Seth, 1969). Although cortisol is the predominant circulating glucocorticoid in primates, corticosterone may comprise a significant or even major fraction of the glucocorticoid bound in brain cell nuclei (Turner, Smith and Carroll, 1979).

The enzymatic pathways for the synthesis of adrenal steroids from cholesterol, which the adrenals may take up from blood or synthesize from acetate, are well known (e.g., Fregly and Luttge, 1982). Briefly, cholesterol undergoes a series of hydroxylations, catalyzed by the mitochondrial desmolase enzyme complex, leading to pregnenolone, which is then converted to progesterone by the actions of the microsomal enzymes

Table 1-1: Adrenal steroid hormone names and abbreviations

IUPAC Names	Abbreviations	Trivial Names
4-Androsten-17β-ol-3-one	T	Testosterone
5-Androsten-3β-ol-17-one	DHEA	Dehydroepiandrosterone
4-Pregnen-3, 20-dione	P_4	Progesterone
4-Pregnen-11α, 17α, 21-triol-3, 20-dione	11α-F	Epicortisol
4-Pregnen-11β, 17α, 21-triol-3,20-dione	F	Cortisol
4-Pregnen-11β,21-diol-3,20-dione	B	Corticosterone
4-Pregnen-11β,21-diol-3,18,20-trione	Aldo	Aldosterone
4-Pregnen-17α-ol-3,20-dione	17α-OH-P_4	17α-Hydroxyprogesterone
4-Pregnen-17α,21-diol-3,20-dione	DOF, S	Cortexolone
4-Pregnen-21-ol-3,20-dione	DOC, 11-DOC	Desoxycorticosterone
5-Pregnen-3β-ol-20-one	Pg	Pregnenolone
1,4-Pregnadien-9-fluoro-11β,16α,17α, 21-tetrol-3,20-dione 16, 17-acetonide	TA	Triamcinolone Acetonide
1,4-Pregnadien-9-fluoro-16α-methyl-11β, 17α,21-triol-3,20-dione	DEX	Dexamethasone

3β-hydroxy steroid dehydrogenase and 3-ketosteroid isomerase. Some progesterone is converted to 17α-OH-progesterone by the cytoplasmic 17 α-hydroxylase. Progesterone and 17α-OH-progesterone are converted by a cytoplasmic C-21 hydroxylase to 11-deoxycorticosterone and 11-deoxy-cortisol, respectively. A mitochondrial 11β- hydroxylase enzyme finally converts 11-deoxycorticosterone and 11-deoxycortisol to the finished glucocorticoid products corticosterone and cortisol. The adrenal cortex does not store its secretions, but it does store an abundance of the precursor cholesterol in lipid droplets. The adrenal stimulators adrenocorticotropic hormone (ACTH) and angiotensin II, by processes involving cyclic AMP, accelerate the conversion of cholesterol to pregnenolone, the rate-limiting step in adrenal steroidogenesis. This conversion is rapidly followed by the synthesis and secretion into the circulation of the active corticosteroids.

Glucocorticoids are so named for their role in regulating glucose metabolism. They act directly on most tissues, and indirectly influence all tissues. An early effect of glucocorticoids is the inhibition of glucose uptake by adipose tissue, skin, fibroblasts, and lymphoid tissue. There is a decrease in macromolecular (protein, lipid, and nucleic acid) synthesis and an increase in protein degradation in these tissues and in muscle. There is an increase in lipolysis in fat cells, and a depletion of glycogen in muscle. These catabolic actions result in the release of amino acids, free fatty acids, glycerol, and nucleotides into the circulation. In contrast, the actions of glucocorticoids in the liver are primarily anabolic, resulting in a general increase in RNA and protein synthesis, as well as the specific

induction of a number of enzymes. The amino acids derived from peripheral catabolism are substrates for increased glucose formation (gluconeogenesis) in liver, and to a lesser extent in the kidney. Glycogen accumulates in liver, and blood glucose levels tend to rise. The latter change induces a compensatory increase in insulin, which counteracts many of the glucocorticoid effects.

Certain tissues are spared from the catabolic actions of glucocorticoids - brain, red blood cells, heart, liver, kidney. These tissues, perhaps more essential than others, may rather enjoy the additional circulating glucose diverted from elsewhere or produced by gluconeogenesis. The diverse actions of glucocorticoids may thus make teleological sense as a coordinated mechanism for making glucose maximally available to certain essential tissues during and immediately following periods of environmental challenge or stress.

Glucocorticoids also have many diverse effects not related to glucose metabolism. They act at multiple sites to suppress inflammatory and allergic reactions, including inhibition of extravasation and migration of leucocytes, edema, and phagocytosis, decrease in the circulating lymphocytes and eosinophils, involution of the thymus, lymph nodes and spleen, and decrease in antibody production. The lungs respond to glucocorticoids with enhanced catecholamine sensitivity, bronchodilation, and decreased vascular resistance. There may be anabolic effects in the development of some organs, for example the induction of surfactant secretion in the fetal lung. Glucocorticoid actions have been reviewed comprehensively by Baxter and Rousseau (1979). The effects of corticosteroids on the nervous system and

behavior have also been reviewed recently (Bohus, de Kloet and Veldhuis, 1982; Rees and Gray, 1983).

Regulation of Glucocorticoid Secretion

Glucocorticoids are released from the adrenals in response to ACTH, which is in turn secreted by cells of the anterior pituitary in response to corticotropin releasing hormone (CRH), of hypothalamic and perhaps extrahypothalamic origin (Sayers and Portanova, 1975; Vale, Spiess, Rivier and Rivier, 1981). Three factors are known to control glucocorticoid secretion: stress, rhythms, and corticosteroid feedback.

The pituitary-adrenal system responds within a few minutes to a wide variety of noxious stimuli, termed stressors. The "general adaptation syndrome" (Selye, 1950) produced by this pituitary-adrenal stress response is both nonspecific with respect to a variety of stimuli and relatively slow to develop, in contrast to the autonomic responses which can produce relatively stimulus-specific and extremely rapid adaptive changes in many organ systems. Although a distinction is frequently drawn between "physiological" stressors (e.g., trauma, hemorrhage, hypoxia, infection, ether, cold, heat, fasting), and less noxious "psychological" stressors (e.g., immobilization, handling, mild electric shock, loud noise, and situations that produce fear, guilt, anxiety, or frustration), this distinction is often blurred in practice. When pain, discomfort, and emotional reactions were avoided carefully, several "physiological stressors" (fasting, heat, exercise) no longer elevated corticosteroid levels. This finding led Mason (1971) to suggest that the essential property of all stressors may be the ability to elicit a behavioral response of emotional arousal or hyperalerting,

which prepares the organism for flight, struggle, or strenuous exertion in a threatening situation.

The circadian rhythm in glucocorticoid secretion appears to be entrained by the organism's rest-activity cycle. The secretory peak occurs just before the active phase, even when the relation of activity to the lighting cycle is reversed, as in humans working on night shifts, or in rats fed only during the day (e.g., Morimoto, Arisue and Yamamura, 1977). In addition to the circadian rhythm, higher frequency oscillations in corticosteroid secretion have been revealed by frequent sampling (every 20 minutes). Recent evidence (Holaday, Martinez and Natelson, 1977) has shown that this pulsatile secretion, previously regarded as "episodic," actually follows an ultradian rhythm (frequency greater than one cycle in 24 hours), having a predominant periodicity of about 90 minutes and other components harmonically related to the circadian rhythm. These rhythmic fluctuations in plasma cortisol were synchronized among 8 isolated, restrained, undisturbed monkeys, indicating their entrainment by environmental factors such as the feeding or lighting schedule. The unexpected finding that ultradian cortisol rhythms were not disrupted by infusion of supramaximal ACTH challenges the classic concept that periodic bursts of corticosteroid output depend entirely on the immediately preceding release of ACTH. The physiological function of corticosteroid rhythms is unknown.

ACTH secretion is regulated by two temporally distinct negative feedback mechanisms, a rate-sensitive fast feedback (FFB), which occurs 5 to 30 minutes after steroid administration, and a proportional, delayed feedback (DFB), which appears after one or more hours (Dallman and Yates, 1969; Jones, Hillhouse and Burden, 1977). These two phases

are separated by a "silent" period, during which negative feedback is not observed. Differences in the steroid structure-activity relationships for FFB and DFB indicate that different receptor mechanisms may be involved.

There is evidence that corticosteroid feedback actions may be exerted at multiple sites. The sensitivity of ACTH-secreting pituitary cells to inhibition by physiological doses of natural and synthetic corticosteroids has been established clearly by studies in which the possibility of hypothalamic involvement was circumvented and by studies of pituitary cells in vitro (Kendall, 1971; Jones et al., 1977). Hypothalamic tissue in vitro also showed a FFB effect of corticosterone, due to decreased release of CRH (Jones et al., 1977). A series of studies (e.g., Feldman and Conforti, 1980) demonstrated that posterior hypothalamic deafferentation, dorsal fornix section, or dorsal hippocampectomy reduced the inhibitory DFB effect of the synthetic fluorinated glucocorticoid, dexamethasone on both basal and ether stress-induced corticosterone secretion in the rat. These findings suggest that the dorsal hippocampus also participates in the feedback regulation of pituitary-adrenal function. Furthermore, several studies (e.g., Carsia and Malamed, 1979) have indicated a direct inhibitory effect of corticosterone and cortisol on ACTH-induced corticosteroidogenesis, suggesting that the self-suppression of adrenocortical cells by end products may provide an additional fine adjustment of steroidogenesis. What remains to be determined is the relative physiological importance of glucocorticoid feedback at the various sites--anterior pituitary, hypothalamus, extrahypothalamic structures, and the adrenal cortex itself. Although it appears that dexamethasone

may act primarily at the pituitary level (Sakakura, Yoshioka, Kobayashi and Takebe, 1981), the pituitary may be less responsive to natural glucocorticoids. One explanation for this difference is that anterior pituitary cytosol contains a transcortin-like macromolecule, which like plasma transcortin binds corticosterone but not dexamethasone and has negligible affinity for DNA-associated acceptor sites in the nucleus (Koch, Lutz, Briaud and Mialhe, 1976). Thus, while the transcortin-like binders cannot interfere with the action of dexamethasone, they can, by competing with the "true" cytoplasmic glucocorticoid receptors, reduce the amount of corticosterone able to interact with these receptors. Such a mechanism might insure that under non-stress conditions the pituitary glucocorticoid receptors would not be occupied, thus allowing them to function only in response to much higher, stress-induced levels of corticosterone.

Glucocorticoids may also act directly upon the hypothalamus and pituitary to modulate the production or secretion of hormones other than CRH and ACTH, such as TRH, TSH, and GH (e.g., Burger and Patel, 1977).

The "compensatory" hypertrophy of the remaining adrenal following unilateral adrenalectomy was long considered a result of decreased corticosteroid feedback. However, compensatory adrenal growth was recently shown to be neurally rather than hormonally mediated, dependent on reciprocal neural connections between the hypothalamus and adrenal (Dallman, Engeland and Shinsako, 1976).

While much progress has been made in elucidating individual factors influencing pituitary-adrenal activity (neural input, feedback, stress, rhythms), there is still a significant gap in our understanding of how these isolated components function together in the intact organism.

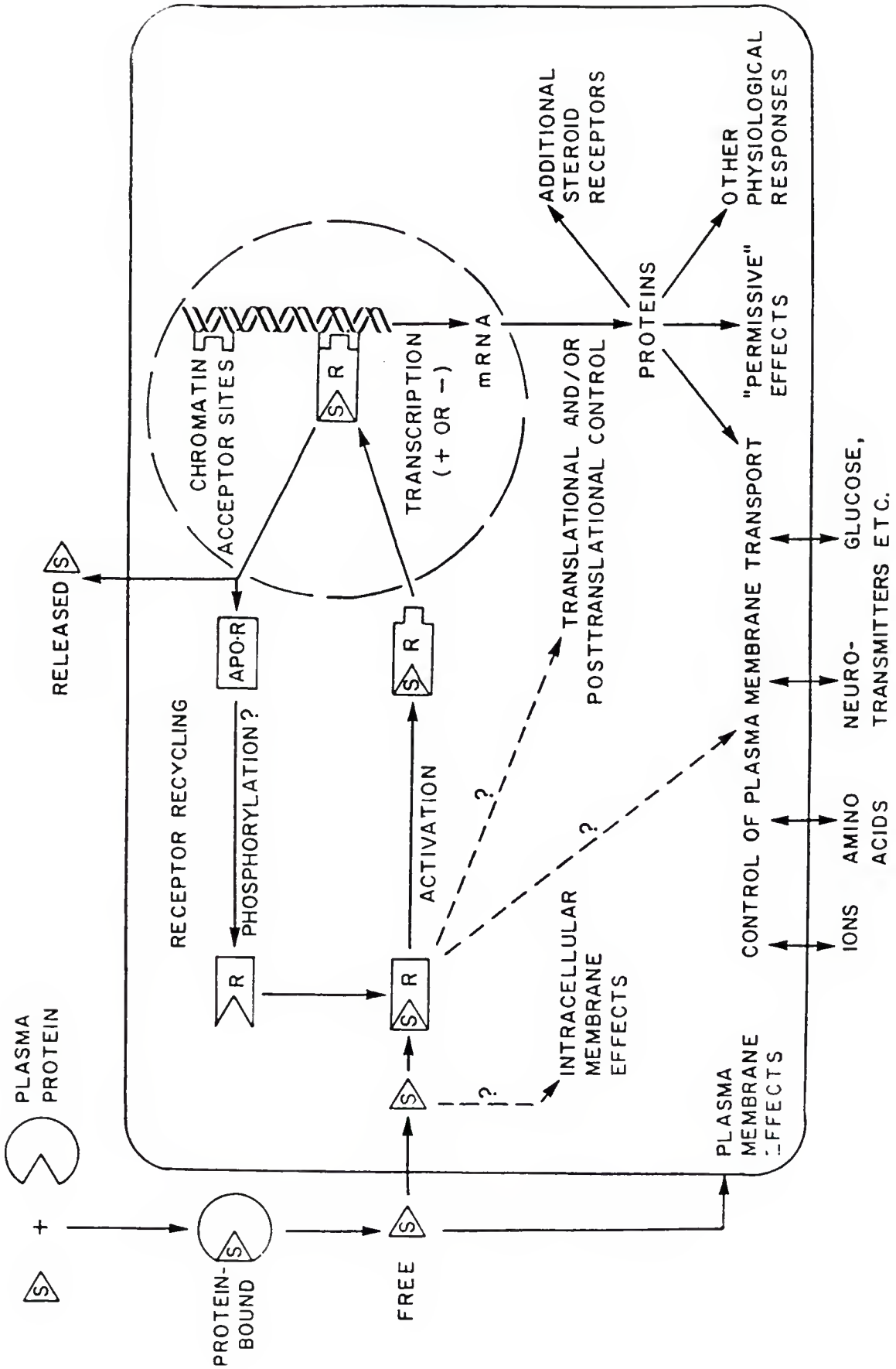
Overview of Corticosteroid Mechanisms

Many of the effects of corticosteroids are believed to be mediated by interactions of the steroid molecules with steroid-specific cytoplasmic macromolecular receptors which concentrate as hormone-receptor complexes in the target cell nuclei, where they initiate the alterations in specific RNA and protein metabolism that then lead to the ultimate physiological, neuroendocrine and behavioral effects. Other less well-understood steroid effects may result from direct interactions of the steroid molecule with components of target cell membranes. Some established and hypothetical events in corticosteroid action are represented in Fig. 1-1.

Although the natural adrenal steroids are soluble enough to be transported unassisted in plasma, about 75% of the circulating glucocorticoid (cortisol or corticosterone) is bound to an α-globulin called transcortin or corticosteroid-binding globulin (CBG), about 15% is bound to serum albumin, and only about 10% is free (Westphal, 1975). Plasma transcortin does not seem to be necessary in any way for the biological activity of the steroids; current evidence supports the dogma that only the free steroid in the plasma can exert physiological action. Although the brain capillary transit time is too short in relation to the dissociation rate or half-life ($t_{1/2}$) of the CBG-glucocorticoid complex to allow the uptake of CBG-bound cortisol and corticosterone, the albumin-bound corticosteroids dissociate rapidly enough to be available for transport through the blood-brain barrier (Pardridge, 1981). (Liver capillary transit time and membrane permeability are

Fig. 1-1. Some established and hypothetical steps in corticosteriod action. S = steroid molecule, R = receptor, APO-R = aporeceptor.

Different shapes of R represent different conformations or covalent modifications. The broken lines indicate hypothetical mechanisms or loci of hormone action.


PLASMA PROTEIN
+
S
PROTEIN-BOUND
S
FREE
S
PLASMA MEMBRANE EFFECTS
?
INTRACELLULAR MEMBRANE EFFECTS
?
?
CONTROL OF PLASMA MEMBRANE TRANSPORT
IONS
AMINO ACIDS
NEURO-TRANSMITTERS
GLUCOSE, ETC.
RECEPTOR RECYCLING
R
PHOSPHORYLATION?
APO:R
RELEASED
S
S R
ACTIVATION
S R
CHROMATIN ACCEPTOR SITES
S R
TRANSCRIPTION (+ OR −)
mRNA
TRANSLATIONAL AND/OR POSTTRANSLATIONAL CONTROL
PROTEINS
ADDITIONAL STEROID RECEPTORS
OTHER PHYSIOLOGICAL RESPONSES
"PERMISSIVE" EFFECTS

greater, allowing uptake of both albumin and CBG-bound glucocorticoids.) Transcortin does reduce the amplitude of free glucocorticoid variations in response to large rhythmic or stress-induced changes in adrenal output. The potent synthetic fluorinated glucocorticoids (dexamethasone and triamcinolone), as well as the natural mineralocorticoid aldosterone, are only very weakly bound by transcortin. Since the natural glucocorticoids can significantly occupy mineralocorticoid receptors when present in high concentrations, and since the total concentration of plasma glucocorticoids is much greater than the normal concentration of aldosterone, the "buffering" effect of glucocorticoid binding by transcortin is apparently necessary to prevent the saturation of mineralocorticoid receptors by glucocorticoids (Funder, Feldman and Edelman 1973). It is not known why the glucocorticoid/mineralocorticoid ratio is so large, requiring this rather peculiar mechanism to confer specificity of hormone action.

It is often assumed that target cell membranes do not present a barrier to free lipophilic steroids, and that their passage into the target cell is governed solely by simple diffusion. Recent studies have, however, demonstrated for at least several different cell types (isolated rat liver and pituitary cells and ACTH-secreting mouse pituitary tumor cells) that glucocorticoid passage through the plasma membrane may involve carrier-mediated transport in addition to simple diffusion (e.g., Harrison, Fairfield and Orth, 1977; Koch, Sakly and Lutz-Bucher, 1981). It is not yet clear how general this phenomenon may be in terms of other target cells and hormones.

It has been observed that some steroids may have several different actions in the nervous system that are mediated independently by their different metabolites, but there is no evidence that the metabolites of the natural glucocorticoids corticosterone and cortisol are functionally important and possess their own non-enzymatic high-affinity binding sites in brain or pituitary. Following in vivo injections of [^{3}H]corticosterone the radioactivity extracted with methylene chloride from the nuclear fraction of rat brain was found to consist of approximately 90% authentic (isochromatographic) corticosterone (McEwen, Magnus and Wallach, 1972). Further investigation of glucocorticoid metabolism in brain tissue is required, however, since acid metabolites of cortisol possessing different, specific biological activities (as enzyme inducers) have recently been found in rat liver (Voigt and Sekeris, 1980).

The cytoplasmic steroid receptors are thermolabile proteins with stereo-specific binding sites. Before the steroid can bind to the receptor with high affinity ($K_d \simeq 10^{-9}$M), the corticosteroid receptor protein ("aporeceptor") may be required to undergo an energy-dependent transformation (possibly a phosphorylation) in order that the potential binding site may be "switched on" to the appropriate conformation for interaction with the steroid. A rapid "switching-off" or down-regulation of the steroid-binding sites (possibly mediated by a phosphatase) has also been observed, suggesting that cells may utilize an internal phosphorylation-dephosphorylation feedback cycle to modulate physiological responses by regulating the amount of receptor capable of interacting with free steroid. Thus, under many circumstances a substantial pool of latent or "cryptic" aporeceptors may be present in

many target cells. This dynamic regulation of the hormone binding site itself has only recently been explored in cells derived from a few peripheral tissues (e.g., Sando, Hammond, Stratford and Pratt, 1979), and is an intriguing area for brain studies (e.g., Luttge, Densmore and Gray, 1982). The number of receptors capable of interacting with free steroid may be subject to additional regulation by certain proteolytic enzymes that can disconnect the steroid binding site from the region of the receptor molecule that contains the nuclear binding site, resulting in non-functional steroid-binding fragments termed "mero-receptors" (e.g., Niu, Neal, Pierce and Sherman, 1981).

After binding, the non-covalent cytoplasmic steroid-receptor complex must next undergo a transformation that results in the development of a high affinity for certain nuclear components associated with the genome. This process of "activation" probably involves a steroid-induced conformational change in the acidic receptor protein which brings a positively-charged "acceptor" binding site to the surface of the molecule (e.g., Barnett, Schmidt and Litwack, 1980). The nuclear "acceptors" to which the activated receptor complexes now bind are unidentified components of chromatin (possibly non-histone proteins) that possess high-affinity and, to varying extents, tissue- and receptor-specific binding domains. Although the acceptors are probably not merely specific DNA nucleotide sequences, they do appear to regulate the interactions of the steroid-receptor complexes with the DNA (e.g., Bugany and Beato, 1977; Cidlowski and Munck, 1980).

Following the formation of the ternary steroid-receptor-acceptor complex the chromatin structure becomes altered in subtle ways that lead to changes in the rates of transcription of specific mRNA species (e.g.,

Johnson, Lan and Baxter, 1979). These specific mRNA molecules are then translated to produce the proteins that mediate the hormone-induced physiological responses. Fig. 1-1 indicates that the proteins whose rates of synthesis are modulated by the steroid may encompass a broad spectrum of cellular functions: additional steroid receptors; components or modulators of membrane transport mechanisms; enzymes of intermediary metabolism; protein kinases, components of peptide hormone- or neurotransmitter-sensitive receptor-adenylate cyclase complexes, and other modulators whose altered synthesis may contribute to the so-called "permissive" effects of steroids; and even specific proteins required for some catabolic steroid effects (e.g., thymus involution) are all examples of proteins that may be regulated to produce the ultimate steroid response (e.g., Baxter and Rousseau, 1979).

After exerting their genomic effect, the receptors are either degraded or recycled back to their unbound, nonactivated cytoplasmic form by a process that may be linked to cell metabolism by a requirement for ATP (e.g., Aronow, 1978). The nuclear "processing" of the receptors, the "off-reaction," and receptor recycling are understood very poorly; it is possible that some steroid dissociation may occur before the receptors are released from their chromatin acceptor sites, and there are hints that the process may be coupled to the proposed cyclic transformations of the steroid binding sites. The released steroid molecules may either re-enter the receptor cycle or diffuse out of the cell into the circulation to enter another cell or to be metabolized and excreted.

Fig. 1-1 also indicates several largely unexplored potential mechanisms of steroid influence on cellular function that do not

directly involve events at the genome. The suggestions that corticosteroid-receptor complexes may directly exert translational (e.g., Kulkarni, Netrawali, Pradhan and Sreenivasan, 1976) or post-translational (e.g., Trajkovic, Ribarac-Stepic and Kanazir, 1974) control over specific protein synthesis or that they may directly regulate membrane transport mechanisms are hypothetical at present. The suggestion that some glucocorticoid effects may result from the interaction of free steroids with intracellular membrane systems is also hypothetical (for review, see Nelson, 1980); glucocorticoids are known to modify some membrane properties, but no functional consequences of such changes are yet well established. Free steroids may also exert important effects at the cell plasma membrane; these include rapid, steroid-specific changes in the firing rates of some neurons (for review, see Feldman, 1981; McEwen, David, Parsons and Pfaff, 1979).

Since a steroid's affinity for the cytoplasmic receptors does not adequately predict the magnitude of the physiological response, it is necessary to classify all steroids into one of four categories on the basis of their physiological effectiveness (Rousseau, Baxter and Tomkins, 1972). Optimal inducers are steroids that all produce the same maximal response when present in saturating amounts. For example, aldosterone will produce as great a glucocorticoid response as dexamethasone (in many tissues) when present in very high concentrations. Suboptimal inducers elicit smaller, less-than-maximal responses even when present in saturating concentrations; 11-deoxycorticosterone is an example of a suboptimal glucocorticoid. Anti-inducers or antihormones produce no typical physiological responses by themselves, but rather behave as competitive inhibitors of the active

hormones; progesterone and cortexolone (11-deoxycortisol) are antiglucocorticoids. Finally, inactive steroids do not bind to the specific steroid receptors at all. It should be stressed that the classification of a particular steroid must refer to a specific, measurable response and may vary among species and from one tissue to another.

It is believed that different ligands can promote different degrees of conformational change, leading to the formation of steroid-receptor complexes with different states of "partial activation" (different affinities for nuclear acceptor components). Munck and Leung (1977) have proposed that each relevant steroid or class of steroids binds to the receptor and promotes a subsequent conformational change that differs in degree from that produced by other steroids. Optimal inducers produce the highest degrees of activation, and anti-inducers either do not promote activation or promote minimal, ineffective increases in affinity for nuclear acceptors. Suboptimal inducers produce intermediate states of activation. Other models of agonist and antagonist interactions with the glucocorticoid receptor are also under active consideration (Rousseau and Baxter, 1979; Sherman 1979).

Anatomical Distribution of Corticosteroid Binding

Neuronal nuclear concentration of [^{3}H]corticosterone has been demonstrated by autoradiography in structures of the limbic system, brain stem, and spinal cord, but not in the hypothalamus (McEwen, Gerlach and Micco, 1975; Stumpf and Sar, 1975; Warembourg, 1973). In adrenalectomized rats, nuclear accumulation of [^{3}H]corticosterone was most intense in structures related to the hippocampus, including the

postcommissural hippocampus, dentate gyrus, induseum griseum (supracallosal hippocampus), anterior (precommissural) hippocampus, and subiculum. Strong nuclear labeling was also seen in the lateral septum, amygdala (cortical, central, and basomedial nuclei), and the piriform, entorhinal, suprarhinal, and cingulate cortices. Additional labeling, although weaker and less frequent, was present in the anterior olfactory nucleus, medial amygdaloid nucleus, habenula, red nucleus, and subfornical organ. Motor neurons in cranial nerve nuclei and spinal cord were strongly labeled, and some glial cells were weakly labeled (Stumpf and Sar, 1979). The pattern of in vivo uptake of [^{3}H]corticosterone determined by autoradiography (highest in hippocampus and septum, followed by amygdala, cortex and hypothalamus) agrees well with the anatomical distribution of cytoplasmic [^{3}H]corticosterone binding sites (McEwen et al., 1972; Grosser, Stevens and Reed, 1973); and with the patterns of [^{3}H]corticosterone binding found in purified nuclei both following [^{3}H]hormone injections in vivo (McEwen, Weiss and Schwartz, 1970) and after incubation of brain slices with [^{3}H]corticosterone in vitro (McEwen and Wallach, 1973; de Kloet, Wallach and McEwen, 1975). Corticosterone target cells have been observed in the anterior pituitary of the rat (Warembourg, 1973) and Pekin duck (Rhees, Abel and Haack, 1972), but not the rhesus monkey (Pfaff, Gerlach, McEwen, Ferin, Carmel and Zimmerman, 1976).

The distribution of target cells for [^{3}H]cortisol in the brains of adrenalectomized rats was identical to that for [^{3}H]corticosterone (Stumpf and Sar, 1973). Nuclear binding sites for cortisol appeared to be saturated by endogenous corticosteroids in adrenally intact mice (Schwartz, Tator and Hoffman, 1972) and guinea pigs (Warembourg, 1973).

The synthetic glucocorticoid dexamethasone displayed a surprisingly different pattern of uptake from that of natural glucocorticoids. Whereas corticosterone and cortisol were concentrated strongly by neurons, [^{3}H]dexamethasone was accumulated weakly by all types of cells in the brain (Rees, Stumpf and Sar, 1975; Rhees, Grosser and Stevens, 1975). The labeling was heaviest in epithelial cells of the choroid plexus and ventricular lumen, and was also observed in vascular endothelial cells, glia, meninges, ependyma, circumventricular organs, and in neurons in areas near the third ventricle (preoptic area, hypothalamus, thalamus) and lateral ventricle (septum, caudate, amygdala). In contrast to [^{3}H]corticosterone, [^{3}H]dexamethasone was concentrated only very weakly by hippocampal neurons. Furthermore, the presence of endogenous adrenal hormones in intact rats did not affect the pattern of [^{3}H]dexamethasone localization (Rhees et al., 1975). In the pituitary, dexamethasone was concentrated heavily by cells in the pars distalis (particularly corticotrophs) and pars nervosa, but not pars intermedia (Rees, Stumpf, Sar and Petrusz, 1977). The autoradiographic data were consistent with the pattern of in vivo uptake of [^{3}H]dexamethasone revealed by direct measurements of tissue radioactivity (de Kloet, van der Vies, and de Wied, 1974).

The strikingly different patterns of distribution of these natural and synthetic glucocorticoids have been interpreted as evidence for the existence of at least two classes of glucocorticoid receptors differing in their distribution and steroid specificity. However, some of the findings may be explained without reference to the concept of receptor heterogeneity. There are large differences in the permeability of the

blood-brain barrier to different steroids (Pardridge and Mietus, 1979). Dexamethasone appears to enter the brain more slowly than corticosterone; time course studies showed that maximal binding in hippocampal cell nuclei occurred one hour after injection of [^{3}H]corticosterone, but two hours after [^{3}H]dexamethasone (de Kloet et al., 1975). Similarly, the cellular accumulation of [^{3}H]dexamethasone in the hippocampus seen autoradiographically three hours after injection was not yet evident at 30 minutes (Rees et al., 1975). The greater blood-brain barrier to dexamethasone may also explain some discrepancies between the patterns of nuclear binding of glucocorticoids obtained in vivo and in slices incubated in vitro. Although the in vivo nuclear binding of [^{3}H]corticosterone in hippocampus was more than ten times that of [^{3}H]dexamethasone, this difference was dramatically reduced (corticosterone: dexamethasone ratio of 1.2 - 1.5) when slices of hippocampus were incubated with the steroids in vitro (de Kloet et al., 1975; McEwen, de Kloet and Wallach, 1976). It is possible that the small but significant remaining differences in nuclear binding observed in the in vitro slice experiments (i.e. greater binding of corticosterone in hippocampus and of dexamethasone in hypothalamus and pituitary) may have resulted from factors other than glucocorticoid receptor heterogeneity: for example, differences in the rates of cellular penetration of the two steroids which may persist in the slice experiments, and differences in the relative abilities of the two steroids to promote activation and nuclear binding of the steroid-receptor complexes (e.g., Svec and Harrison, 1979).

Characterization of Soluble Corticosteroid Receptors in Brain

The natural glucocorticoids, corticosterone and cortisol; the synthetic glucocorticoids triamcinolone acetonide (TA) and dexamethasone; and the natural mineralocorticoids, aldosterone and 11-deoxycorticosterone (DOC), bind to steroid-specific, saturable brain cytosol components believed to be the physiological transducer molecules or "receptors." One goal of receptor research is to identify the different classes or categories of adrenal steroid action in the brain and to study individually the receptors mediating these actions. The categories "glucocorticoid" and "mineralocorticoid" are defined by distinguishable peripheral physiological effects and steroid specificities; this distinction may be meaningful in the nervous system, but it should not be assumed a priori that brain steroid effects and specificities closely correspond to those of other organ systems.

In comparison with the corticosteroid receptors found in other tissues, the few reported physicochemical properties of brain receptors are generally unremarkable. They have been distinguished from those of transcortin by a number of criteria; unlike transcortin, the brain cytoplasmic glucocorticoid binding protein was found to bind the synthetic steroids dexamethasone and TA with high affinity and to possess sulfhydryl groups whose modification led to the loss of functional steroid binding sites (e.g., Chytil and Toft, 1972; McEwen and Wallach, 1973).

The resolution of the different classes of brain corticosteroid receptors is confusing because it involves two distinct but related issues: the possible existence of separate binding sites for natural and synthetic glucocorticoids; and the distinction between

glucocorticoid and mineralocorticoid binding sites. The principal technique for defining distinct binding sites is the in vitro measurement of steroid specificity in competition experiments. Oddly, the actual affinities of different competing steroids for brain cytosol [^{3}H]steroid binding sites have seldom been reported. Specificity data have typically been reported only as a rank ordering of the abilities of different steroids to compete for the binding of a given [^{3}H]steroid.

Although both natural and synthetic agonists were bound with similar high affinities by brain cytosol (Chytil and Toft, 1972), dexamethasone did not reduce the binding of [^{3}H]corticosterone in whole brain cytosol to the extent that was predicted from its physiological potency as a peripheral glucocorticoid (Grosser et al., 1973; McEwen and Wallach, 1973). In most studies, corticosterone and dexamethasone were equally effective in competition for [^{3}H]dexamethasone and [^{3}H]TA binding (e.g., Chytil and Toft, 1972; Stevens, Reed and Grosser, 1975; de Kloet and McEwen, 1976).

Comparative measurements of the total cytosol binding capacity for corticosterone and dexamethasone have been reported. Because a number of poorly understood variables (such as the composition of the incubation buffer, the presence or absence of phosphatase inhibitors, the time elapsed between tissue homogenization and cytosol labeling with [^{3}H]steroid, etc.) have not yet been fully explored or controlled, published estimates of apparent binding capacity (B_{max}) are often in conflict. The discovery by de Kloet et al. (1975) that the spontaneous loss of [^{3}H]dexamethasone binding capacity from unlabeled cytosol was more rapid than the loss of [^{3}H]corticosterone binding sites, stimulated experiments in which tissues were homogenized in the presence of the

[^{3}H]steroids. With this alteration in methodology, binding capacities for [^{3}H]dexamethasone and [^{3}H]corticosterone in hippocampal cytosol were found to be equal, and hypothalamic cytosol had an even slightly higher capacity for [^{3}H]dexamethasone than for [^{3}H]corticosterone (e.g., de Kloet et al., 1975; Turner and McEwen, 1980).

High affinity mineralocorticoid (type I) binding sites, which occur in significant concentrations principally in the hippocampus and associated structures, have been studied in rat brain cytosol (Anderson and Fanestil, 1976; Moguilewsky and Raynaud, 1980). The mineralocorticoid receptors have high affinity for aldosterone and DOC and, surprisingly, an almost equally high affinity for progesterone. Since [^{3}H]aldosterone, [^{3}H]corticosterone and [^{3}H]dexamethasone all bind (with different affinities) to both glucocorticoid and mineralocorticoid brain receptor sites, it has been most productive to study the binding of [^{3}H]aldosterone in the presence of an excess of the "pure" glucocorticoid R26988 (Moguilewsky and Raynaud, 1980). Although concentrations of glucocorticoid and mineralocorticoid receptor sites were comparable in hippocampal cytosol, the concentration of high-affinity mineralocorticoid binding sites was much lower than the concentration of glucocorticoid binding sites in whole brain.

Although the discrepancies between in vivo and in vitro (brain slice) nuclear binding of [^{3}H]corticosterone and [^{3}H]dexamethasone can be explained largely without reference to the possible heterogeneity of unbound glucocorticoid receptors, several observations (such as the relatively poor ability of dexamethasone to compete for [^{3}H]corticosterone binding sites) have suggested that natural and synthetic glucocorticoids may bind to somewhat different receptor populations.

When cytosol samples were chromatographed on DEAE-cellulose ion-exchange columns, complexes formed with [^{3}H]corticosterone and [^{3}H]dexamethasone were eluted as multiple peaks, and the proportion of bound [^{3}H]steroid in each of the two major peaks differed for the two glucocorticoids (de Kloet and McEwen, 1976). Although it is unlikely that the two major peaks of both hippocampal [^{3}H]dexamethasone and [^{3}H]corticosterone binding merely represent different pools of activated and nonactivated receptors, it is quite possible that they are distinct proteolytic fragments (created in vitro) of a single larger intact glucocorticoid receptor. Affinity chromatography of rat brain cytosol on columns of immobilized deoxycorticosterone (DOC) hemisuccinate (subsequently eluted with [^{3}H]corticosterone) selectively purified one of the two major [^{3}H]corticosterone binding peaks resolved by ion exchange chromatography (de Kloet and Burbach, 1978).

Apparent receptor heterogeneity was also observed in rat brain (and pituitary) cytosol following isoelectric focussing of labeled samples on polyacrylamide gels (MacLusky, Turner and McEwen, 1977). In brain cytosol three major specific [^{3}H]corticosterone binding peaks were resolved; these had isoelectric points (pIs) of approximately 6.8, 5.9 and 4.3. When [^{3}H]dexamethasone was the ligand only two peaks were found (the peak at pI 4.3 was absent). Furthermore, the relative sizes of the two remaining peaks were different for the two ligands. Wrange (1979) has suggested that the apparent receptor heterogeneity observed by MacLusky et al. (1977) may have resulted from proteolytic artifacts and that the brain cytosol [^{3}H]cortiscosterone binding peak at pI 4.3 (reported by the same workers) can probably be attributed to residual transcortin (CBG) remaining in the tissue following incomplete

perfusion. Wrange found only a single peak of radioactivity (at pI 6.1) when rats were extensively perfused and hippocampal cytosol samples labeled with either [^{3}H]corticosterone or [^{3}H]dexamethasone were analyzed. However, it was not possible to conclude that CBG or a class of CBG-like binding sites was definitely not present, since free steroid was removed from the samples prior to the relatively long focussing procedure, which would have both allowed extensive dissociation of steroid from the CBG and eventually denatured the steroid binding sites as they entered the region of low pH. Wrange also found that limited tryptic digestion of hippocampal cytosol labelled with either [^{3}H]corticosterone or [^{3}H]dexamethasone produced two peaks of bound radioactivity having pI values of 6.0 and 6.4. These pI values are close enough to those reported by MacLusky et al. (1977) to suggest that proteolytic fragments of a single molecule may have been responsible for the observed heterogeneity. The relative sizes of the two trypsin-induced peaks were different when [^{3}H]dexamethasone was substituted for [^{3}H]corticosterone; this situation may have resulted either from the possession of slightly different trypsin substrate characteristics by the receptor complexes formed with the different steroids or from different rates of dissociation of [^{3}H]corticosterone from the two trypsin-induced receptor fragments.

The autoradiographic data reviewed above have led to the suggestion that neurons contain glucocorticoid receptors that preferentially bind corticosterone and cortisol, whereas glial cells contain glucocorticoid receptors having higher affinity for dexamethasone and TA (e.g., McEwen et al., 1979). There is, however, very little evidence to support this dichotomy. Although dexamethasone is a potent inducer of glycerol-

phosphate dehydrogenase (GPDH) in cultured glial tumor cells, and cytosol prepared from these tumor cells and from optic nerve oligodendrocytes contains high-affinity [^{3}H]dexamethasone binding sites, glial cells also respond to natural glucocorticoids (e.g., Breen, McGinnis and de Vellis, 1978; Cotman, Scheff and Benardo, 1978). Furthermore, there is no evidence (e.g., Clayton, Grosser and Stevens, 1977) that brain [^{3}H]dexamethasone binding capacity increases faster than [^{3}H]corticosterone binding capacity during the period of rapid glial growth associated with myelination. Glial tumor cells were found to contain only one glucocorticoid receptor with a pI of 5.9, corresponding to the molecular species that bound [^{3}H]dexamethasone preferentially in the rat brain (MacLusky, unpublished, cited by McEwen et al., 1979). This observation cannot, however, be considered strong evidence for a neuronal-glial receptor dichotomy, since Wrange (1979) reported a similar isoelectric binding profile containing only a single radioactive peak in hippocampal cytosol from perfused animals. It is possible that different concentrations of proteolytic enzymes in the cytosol samples prepared from brain tissue and from cultured glial cells could explain the differences between the [^{3}H]glucocorticoid binding profiles observed by MacLusky and colleagues in these different preparations. The demonstration that [^{3}H]dexamethasone binding sites disappear from hippocampal cytosol (in the absence of steroid) more rapidly than [^{3}H]corticosterone binding sites (de Kloet et al., 1975) may result from the gradual alteration of a single initial population of steroid binding sites by enzymatic processes that are triggered upon cell disruption and that proceed rapidly in the absence of protective

steroid ligands. This apparent differential loss of free binding sites for [^{3}H]dexamethasone and [^{3}H]corticosterone may also result, at least in part, from the presence of a population of relatively more stable CBG or CBG-like binding sites in the hippocampal cytosol. Clarification of this complex issue must await the purification and comparison of both intact unbound receptors and steroid-receptor complexes.

Physiological Regulation of Corticosteroid Receptors

The ontogeny of the capacity of rat brain cytosol to bind both natural and synthetic glucocorticoids has been studied. Binding of [^{3}H]dexamethasone was very low immediately after birth, but it reached the adult level sooner than [^{3}H]corticosterone binding, which was higher than that of [^{3}H]dexamethasone immediately after birth. Adult levels of [^{3}H]corticosterone binding were similar to those of [^{3}H]dexamethasone in both hippocampal and hypothalamic cytosols (Olpe and McEwen, 1976). Turner (1978) found that the amount of [^{3}H]corticosterone bound by hippocampal nuclei in adrenalectomized rat pups injected with steroid in vivo was very small in comparison with adult levels. Furthermore, the nuclear binding of [^{3}H]corticosterone by hippocampal pyramidal and dentate granule cells as determined by autoradiography was correlated directly with neuronal age; in the neonatal hippocampus the oldest cells revealed the heaviest labeling, whereas newly arrived cells showed little nuclear retention of steroid. Thus, although the aporeceptor proteins may appear much earlier in development, the production of receptors with functional binding sites and the potential for activation to the nucleophilic state may occur relatively late in the differentiation of these neurons.

An age-related decline in corticosterone receptors has been reported in mouse hippocampus (Finch and Latham, 1974) and in rat cerebral cortex (Roth, 1974). Evidence suggests that senescent intracellular biochemical changes rather than cellular losses are responsible for the decline in cortical receptors (Roth, 1976).

The concentration of intracellular corticosteroid binding sites rises in response to steroid deprivation. Adrenalectomy caused a two-stage increase in the nuclear binding of [^{3}H]corticosterone by hippocampus in vivo and in vitro (McEwen, Wallach and Magnus, 1974) and increased glucocorticoid cytosol receptor concentrations (Stevens et al., 1975; Olpe and McEwen, 1976). The apparent receptor content increased rapidly for the first 2 hours after adrenalectomy and then remained at a plateau for about 12 hrs; the second, slower increase began between 12 and 18 hours after adrenalectomy and approached a new plateau after about 3 days. The first, rapid change, which parallels the decline in plasma corticosterone, certainly represents the disappearance of endogenous corticosterone from brain binding sites and may also reflect the "switching-on" of the steroid binding sites of receptors. The interesting long-term increase results from either the synthesis of new receptors on the "switching-on" of previously unobservable "cryptic" aporeceptors.

Both the concentration of endogenous corticosteroids and the occupancy of corticosteroid receptors in the brain vary with changes in plasma steroid levels. Brain glucocorticoid concentrations, which were intermediate between free and total plasma concentrations and therefore possibly equal to the plasma glucocorticoid concentration available for brain uptake (the free + albumin-bound or "BBB-transportable"

concentration) (Carroll, Heath, and Jarrett, 1975), were found to fluctuate in parallel with both basal circadian and stress-induced changes in the plasma steroid concentrations (Butte, Kakihana and Noble, 1976; Carroll et al., 1975). Furthermore, the diurnal and stress-induced increases in plasma corticosterone decreased the in vitro cytosol binding of [^{3}H]corticosterone in all brain regions examined (Stevens, Reed, Erickson and Grosser, 1973). In most brain regions of unstressed animals, glucocorticoid receptor occupancy varies between about 50% at the diurnal trough and approximately 80% at the peak (Stevens et al., 1973; McEwen et al., 1974; Turner, Smith and Carroll, 1978a,b). In contrast to other brain regions, the preoptic and septal areas exhibited a high level of receptor occupancy even during the morning corticosterone minimum, and no increase at the evening peak (Turner et al., 1978a,b). However, all brain regions showed a circadian variation in the total concentration of cytosol [^{3}H]corticosterone binding sites. Furthermore, the same dose of [^{3}H]corticosterone injected into adrenalectomized mice produced higher hippocampal steroid concentrations at different times of the day (Angelucci, Valeri, Palmery, Patacchioli and Catalani, 1980). The peak brain concentrations varied as the normal circadian rhythm, suggesting that a steroid-independent rhythm of receptor concentration may persist in the adrenalectomized animals.

An investigation of the temporal relationship between glucocorticoid nuclear binding and the availability of cytosol binding sites led to the unexpected finding that there was no net depletion of total hippocampal cytosol binding capacity as a result of nuclear translocation 15-60 min after the injection of fully saturating doses of

either [^{3}H]corticosterone or [^{3}H]dexamethasone (Turner and McEwen, 1980). The predicted cytosol receptor depletion was based on hippocampal nuclear uptake measured following the in vivo [^{3}H]steroid injections. Cytosol samples from rats injected with [^{3}H]steroids were incubated in vitro with additional [^{3}H]steroids to determine the maximal cytosol steroid binding capacity, but no depletion of this total capacity as a result of nuclear translocation was ever observed. This investigation also revealed that [^{3}H]corticosterone injected in vivo could occupy no more than 40% of the total cytosol binding sites measured in vitro. These results suggest the existence of a reserve pool of aporeceptors or "cryptic" receptors which can be rapidly converted to the form capable of binding steroids. However, it must not be assumed that these findings are characteristic only of brain tissue; the same glucocorticoid injection produced large differences in cytoplasmic receptor depletion among 6 different rat glucocorticoid target tissues (Ichii, 1981). For example, an injection of dexamethasone that depleted 75% of heart and muscle cytoplasmic receptors depleted only 40% of liver and lung receptors and only 10% of thymic and spleen receptors (brain samples were not included). Much larger injections were able to fully deplete receptors in all 6 tissues.

Neuropeptide effects on functional steroid receptor concentrations have been observed. The increase in [^{3}H]corticosterone binding capacity of rat hippocampal cytosol observed after hypophysectomy combined with adrenalectomy was greater than that after adrenalectomy alone. Both $ACTH_{1-24}$ (steroidogenic) and $ACTH_{4-10}$ (devoid of corticotrophic activity) eliminated the additional increase attributed to hypophysectomy. Furthermore, vasopressin-deficient (Brattleboro

strain) rats were found to have abnormally low hippocampal cytosol [^{3}H]corticosterone receptor levels that could be restored by physiological doses of vasopressin or des-glycinamide^9-arg^8-vasopressin (a behaviorally potent analog having low antidiuretic activity) (de Kloet and Veldhuis, 1980; de Kloet, Veldhuis and Bohus, 1980).

Several observations indicate the possibility of a rapid, dynamic regulation of the availability of brain steroid binding sites. Hippocampal electrical stimulation resulted in increased in vivo uptake of [^{3}H]cortisol into hypothalamic cells and an increase in the proportion of intracellular hormone bound in the nucleus (Stith, Person and Dana, 1976a). A single injection of reserpine into cats resulted (at 16 hrs post injection) in a decreased concentration of cytosol binding sites for [^{3}H]dexamethasone (Weingarten and Stith, 1978). A rapid influence of metyrapone (an inhibitor of adrenal 11β-hydroxylase) on the binding of [^{3}H] cortisol in pig hypothalamic slices incubated in vitro at 37°C has also been reported (Stith, Person and Dana, 1976b). The quantity of [^{3}H]cortisol bound to cytosol components after 30 min was reduced by 50%, and nuclear-bound [^{3}H]cortisol was inhibited by 70%; the mechanism of this inhibition is unknown.

Lesions have been used to explore possible influences of hippocampal afferents and efferents on the concentration of hippocampal glucocorticoid receptors. Transection of the fimbria bilaterally for a duration of 6 or 80 days did not affect either the concentration of hippocampal [^{3}H]corticosterone and [^{3}H]dexamethasone receptors or the increase in this concentration observed following adrenalectomy (Olpe and McEwen, 1976). Furthermore, fimbria transection in 3-day-old rats did not affect the normal ontogenetic increase in hippocampal binding

sites for [^{3}H]dexamethasone and [^{3}H]corticosterone. In contrast, the concentration of hippocampal [^{3}H]corticosterone receptors was elevated 30 days (but not 10 days) following lesions which included the lateral septal nuclei and the precommissural fornix; lesions which included the medial septal and diagonal band nuclei and the main septal projection to the hippocampus did not alter hippocampal receptor concentrations at either 10 or 30 days after the lesions (Bohus, Nyakas and de Kloet, 1978; Nyakas, de Kloet and Bohus, 1979). Following unilateral dorsal hippocampectomy the concentration of [^{3}H]corticosterone binding sites in the contralateral hippocampus was increased by 74% and 41%, respectively, 10 and 20 days after lesioning (Nyakas, de Kloet, Veldhuis and Bohus, 1981). Thus, some hippocampal afferents and efferents may modulate steroid receptor concentrations.

Corticosterone "Membrane Effects" and Receptors

Some corticosteroid effects on the nervous system are not mediated by the mobile cytoplasmic receptors that affect gene expression; these effects may derive from the alteration of membrane properties by the free steroids themselves or may be mediated by specific membrane-associated receptors. Proposed mechanisms for such effects have included the stabilization of lysosomal membranes, which could delay the release of hydrolytic enzymes; alteration of ribosomal attachement to the endoplasmic reticulum, which could modify protein synthesis; and alteration of the binding of calcium to intracellular membranes, which could influence synaptic function (reviewed in Baxter and Rousseau, 1979; Nelson, 1980).

Dexamethasone appeared to elevate tyrosine hydroxylase activity in the superior cervical ganglion of adrenally intact rats by exerting an excitatory pharmacological influence directly on preganglionic cholinergic nerve terminals; very large doses of corticosterone were completely ineffective, and the slowly-developing effect of dexamethasone was abolished by a cholinergic receptor antagonist (Sze and Hedrick, 1979). The effects of synthetic glucocorticoids on cholinergic neurotransmission have been both excitatory and inhibitory. The excitability of cat somatic motoneurons was increased (Riker, Baker and Okamoto, 1975), and the contraction of guinea pig ileum in response to nerve stimulation was decreased (Cheng and Araki, 1978) by the steroids; in both cases the evidence suggested a direct steroid action on cholinergic terminals.

Systemic or local injection of glucocorticoids has been observed to affect the spontaneous activity of neurons in many brain loci (for review, see Feldman, 1981). Injection of cortisol in intact, freely-moving rats rapidly increased spontaneous activity of units in the anterior hypothalamus and mesencephalic reticular formation, and decreased unit activity in the ventromedial and basal hypothalamus (Phillips and Dafny, 1971a,b). Spontaneous activity of basal hypothalamic neurons in completely deafferented hypothalamic islands was also rapidly decreased following systemic injection of either cortisol (Feldman and Sarne, 1970) or dexamethasone in intact rats (Ondo and Kitay, 1972). Similarly, iontophoretic application of dexamethasone onto medial basal hypothalamic neurons in intact rats produced an immediate depression of cell firing (Steiner, Ruf and Akert, 1969). Mesencephalic neurons also responded to direct application of

dexamethasone with a decrease in firing rate (Steiner et al., 1969), in contrast to the increase in firing rate after systemic injection of cortisol reported by Phillips and Dafny (1971a,b). The injection of dexamethasone into the vicinity of the recording electrode rapidly produced a dramatic decrease in hippocampal multiple unit activity (Michal, 1974), but none of 500 hippocampal neurons tested were responsive to iontophoretic application of either cortisol or corticosterone (Barak, Gutnick and Feldman, 1977). It is not known if some of these reported electrophysiological effects of corticosteroids are mediated by specific receptors, but their short latencies suggest that they are direct membrane effects not mediated by changes in gene expression.

The membrane effects of glucocorticoids probably also include the physiologically important fast feedback (FFB) suppression of the release of CRH by hypothalamic neurons. The addition of corticosterone to the in vitro incubation medium blocked the release of CRH produced by the electrical stimulation of sheep hypothalamic synaptosomes (Edwardson and Bennett, 1974), and there is evidence that the FFB action of corticosterone, which is unaffected by a number of pharmacologic agents, may be mediated by a direct stabilizing interaction of the steroid with the membrane of the CRH-secreting cell, which decreases the flux of calcium into its terminals (Jones et al., 1977).

Glucocorticoids regulate the uptake of [^{3}H]tryptophan by isolated brain synaptosomes incubated in vitro with the steroids; corticosterone and dexamethasone at concentrations above 10^{-7}M elevated the maximal rate (V_{max}) of tryptophan transport by a high affinity synaptosomal uptake system from mouse brain (e.g., Sze, 1976). This effect contrasts

sharply with the reversal by glucocorticoids of the increase in the V_{max} of high affinity GABA uptake into rat hippocampal synaptosomes observed after adrenalectomy (Miller, Chaptal, McEwen and Peck, 1978). The latter effect required hormone pretreatment in vivo and was not observed when the synaptosomes were incubated in vitro with glucocorticoids, suggesting that in this case the steroid action was probably mediated by the "classical" mobile receptor pathway.

Synaptic plasma membranes prepared from osmotically-shocked rat brain synaptosomes have been reported to contain specific binding sites for glucocorticoids (Towle and Sze, 1978). Specific, saturable binding of [^{3}H]corticosterone by synaptic membranes was greatest in the hypothalamus, and lower (but approximately equal) levels were found in hippocampus and cerebral cortex. The affinity of corticosterone for the binding sites (Kd $\simeq$ 10^{-7}M) was similar in the three brain regions, and both corticosterone and synthetic glucocorticoids had similar affinities for the membrane sites. Soluble cytoplasmic receptors and synaptic membrane binding sites in brain are characterized by somewhat different properties of thermal stability and resistance to hydrolytic enzyme attack, making unlikely the possibility of artifactual contamination of the membranes by cytoplasmic receptors. Since the affinity of corticosterone for the membrane binding sites (Towle and Sze, 1978) agrees well with the concentration-response relation for the in vitro stimulation of synaptosomal tryptophan uptake by corticosterone (e.g., Sze, 1976), it is possible that the membrane binding sites are involved in the regulation of tryptophan uptake in the brain.

Summary

Glucocorticoids have profound metabolic, neuroendocrine, and behavioral effects in the mammalian brain (e.g., Rees and Gray, 1982). Although some of the less-well-understood effects may result from direct interactions of the steroid with components of target cell membranes, many of the effects are thought to be mediated by interactions of the hormone molecules with steroid-specific cytoplasmic and nuclear macromolecular receptors that concentrate as activated hormone-receptor complexes in the target cell nuclei, where they initiate the changes in gene expression that produce the ultimate physiological effects. The objectives of brain corticosteroid receptor research are to improve our extremely limited understanding of the basic mechanisms of receptor capacitation, activation, nuclear concentration and recycling; to examine whether the receptor systems for corticosteroid hormones in brain (and pituitary) resemble closely those operative in other target tissues; and to determine how the components of the receptor system are altered in the clinically relevant conditions (such as experimentally-induced diabetes, genetically-determined obesity and hypertension, and normal age-related brain senescence) that are correlated with changes in receptor function.

The experiments presented in this dissertation have been designed to examine a number of the specific physicochemical properties of soluble mouse brain glucocorticoid binding sites. The mouse has been chosen for this research for several reasons. Although a few published studies have indicated the existence of receptors in the mouse brain (e.g., Finch and Latham, 1974; Nelson, Holinka, Latham, Allen and Finch, 1976; Angelucci et al., 1980), no basic characterization of the kinetic

and equilibrium binding parameters or the steroid specificity of these receptors has been reported. Although a modest body of literature (reviewed above) concerned with the properties of rat brain glucocorticoid receptors already exists, recent improvements in receptor methodology have led us to believe that many of the published rat brain results are probably questionable, and thus that complete reexamination of the rat brain receptor system must eventually be undertaken. Therefore, we have chosen the mouse primarily for obvious economic reasons, and because it is relatively easy to rapidly perfuse and remove a large number of mouse brains. The decision to use the mouse instead of the rat has had little impact on the choice or design of specific experiments.

Since it is necessary to eliminate both endogenous gonadal and adrenal steroids before killing the animals, females have been used because they can be simultaneously ovariectomized and adrenalectomized through the same incisions. We have used whole mouse brain cytosol because most of the experiments required large amounts of receptor material and since there is no evidence that there are brain regional differences in the physicochemical properties of corticosteroid receptors. Since the use of smaller brain regions would not have enabled us to distinguish between cell types, it was felt that the additional expense and effort required to work with a smaller brain region such as dorsal hippocampus would not be rewarded adequately.

We have used the labeled glucocorticoids [^{3}H]corticosterone, [^{3}H]dexamethasone and [^{3}H]triamcinolone acetonide (cyclic acetal). [^{3}H] Dexamethasone was used to perform both equilibrium and kinetic studies; [^{3}H]corticosterone was employed to examine the possible

contribution of transport proteins to the total pool of [^{3}H]corticosterone binding sites, whereas the nearly-irreversible ligand [^{3}H]triamcinolone acetonide was used for the lengthy procedures examining the size and shape of the receptors. In contrast to many of the older receptor studies with rat brain reviewed above, our experiments have used a buffer containing ingredients that prevent loss of unoccupied binding sites at 0-4°C; used a rapid assay that can both measure association kinetics conveniently and assay rapidly-dissociating binding complexes; considered the possible consequences of the failure to allow adequate time for "equilibrium" incubations at low ligand concentrations; determined steroid specificity by applying mathematically correct procedures to the analysis of competition data; explored the contribution of CBG-like binding sites to the total pool of corticosterone binding sites, and examined binding site sizes and shapes to assess the homogeneity of the in vitro receptor population. Before presenting experimental data we discuss (in chapters II and III) some simple applications of the binding rate equation and propose some improvements to the methods of graphical analysis of equilibrium competition data currently in use.

CHAPTER II

METHODS FOR THE DETERMINATION OF ASSOCIATION AND DISSOCIATION RATE CONSTANTS AND FOR THE ESTIMATION OF TIMES REQUIRED FOR THE ATTAINMENT OF ARBITRARY DEGREES OF APPROACH TO EQUILIBRIUM BY NON-COOPERATIVE, SINGLE SITE LIGAND-RECEPTOR SYSTEMS

Introduction

The rate equation for noncooperative, single site ligand binding systems may be written

$$dB_L/dt = k_a (S_L-B_L)(B_0-B_L)-k_dB_L, \qquad (2\text{-}1)$$

where B_L is the concentration of specifically-bound ligand, S_L is the total ligand concentration, B_0 is the total concentration of binding sites, k_a and k_d are the second-order and first-order association and dissociation rate constants, and t is the time of incubation. The exact solution to this equation gives the value of B_L as a function of time for the given incubation conditions if the rate constants (or the equilibrium dissociation constant, K_{dL}, and one of the rate constants) and the concentration of ligand and binding sites are known (e.g., De Lean and Rodbard, 1979; Vassent, 1974). Thus, if nonspecific binding and loss due to the inactivation of binding sites may be neglected as an approximation, the solution to the rate equation provides an estimate of the time required for any arbitrary degree of approach to the equilibrium value of specific binding. As an example of its utility,

the solution to the rate equation has been used to examine the effect of inadequate incubation time (during which equilibrium was not attained under conditions of low ligand concentration) on measured "equilibrium" dissociation constants (Aranyi, 1979; Yeakley, Balasubramanian and Harrison, 1980). The solution to the rate equation can also provide insight into several superficially paradoxical phenomena, such as the observation that the degree of approach to equilibrium within a given time is not always a monotonically increasing function of ligand concentration (Vassent, 1974). We present a concise derivation of a computationally convenient form of the solution and discuss several of its applications. Methods for the determination of association and dissociation rate constants (not requiring the complete solution to the rate equation) are also discussed.

Theory

Equation (2-1) may be rewritten in the classical Ricatti form as

$$dB_L/dt = k_a B_L^2 - k_a(K_{dL}+S_L+B_0)B_L + k_a S_L B_0, \qquad (2\text{-}2)$$

where k_d has been replaced by $k_a K_{dL}$. At equilibrium $dB_L/dt=0$ and thus (at equilibrium)

$$B_L^2 - (K_{dL}+S_L+B_0)B_L + S_L B_0 = 0. \qquad (2\text{-}3)$$

This may be rewritten as

$$(B_L-P)(B_L-Q) = 0, \qquad (2\text{-}4)$$

where

$$P = (K_{dL}+S_L+B_0-[(K_{dL}+S_L+B_0)^2-4\ S_L B_0]^{1/2})/2 \qquad (2\text{-}5)$$

and

$$Q = (K_{dL}+S_L+B_0+[(K_{dL}+S_L+B_0)^2-4\ S_L B_0]^{1/2})/2. \qquad (2\text{-}6)$$

The smaller root, P, gives the value of B_L at equilibrium. Now equation (2-2) may be rewritten as

$$dB_L/(B_L-P)(B_L-Q) = k_a dt, \qquad (2\text{-}7)$$

which, on integrating, gives

$$k_a t + c = (\ln[(B_L-P)/(B_L-Q)])/(P-Q), \qquad (2\text{-}8)$$

where c is the integration constant determined by the initial conditions. If $(B_L)_0$ denotes the value of B_L at t = 0 then

$$c = (\ln[[(B_L)_0-P]/[(B_L)_0-Q]])/(P-Q). \qquad (2\text{-}9)$$

Upon substituting (2-9) into (2-8) we get

$$t = (\ln[[B_L-P][(B_L)_0-Q]/[(B_L)_0-P][B_L-Q]])/k_a(P-Q), \qquad (2\text{-}10)$$

and thus

$$\exp [k_a t(P-Q)] = [B_L-P][(B_L)_0-Q]/[(B_L)_0-P][B_L-Q]. \qquad (2\text{-}11)$$

Solving for B_L, we eventually find that

$$B_L = [P(d/e) - Q \exp (-ft)]/[(d/e) - \exp (-ft)], \qquad (2\text{-}12)$$

where $d = Q - (B_L)_0$, $e = P - (B_L)_0$, and $f = k_a(Q-P)$. If the initial binding is zero (a common application), then equation (2-12) simplifies to

$$B_L = [1-\exp(-ft)]S_L B_0/[Q-P \exp(-ft)], \qquad (2\text{-}13)$$

since $QP = S_L B_0$. (This form of the solution is computationally convenient because it avoids the generation of large exponentials as t becomes large.)

The relative error or fractional deviation from equilibrium is defined as

$$\varepsilon = \text{absolute value of } [(B_L-P)/P], \qquad (2\text{-}14)$$

and is easily calculated. If equation (2-12) and (2-14) are solved for t (e.g., Vassent, 1974), then an expression giving the time required for an arbitrary degree of approach to equilibrium is obtained:

$$t(\varepsilon) = [1/k_a(Q-P)] \ln ([Q-P(1-\varepsilon)]/ P[1+(Q-P)/e]). \qquad (2\text{-}15)$$

We now examine some applications of the rate equation and its solution. In the following discussions we shall assume that B_0 remains constant with time (unless a deliberate dilution or concentration is performed). Many in vitro steroid receptor preparations do not, however, possess this stability. For example, a frequent observation has been the gradual inactivation or loss, with time, of unoccupied (free) receptor binding sites (e.g., Luttge et al., 1982). If this is the case, then the rate equation (2-1) must be supplemented with the simultaneous inactivation equation

$$d(B_0-B_L)/dt = -k_{in}\ (B_0-B_L) - dB_L/dt, \qquad (2\text{-}16)$$

where k_{in} is an empirical inactivation constant describing a process of simple unimolecular decay of the normal binding site conformation. (This assumption has, of course, no theoretical basis; it is merely a statement of the observation that the relative early regions of slow receptor inactivation curves may be approximately fit to simple exponentials.) Equation (2-16) simplifies to

$$dB_0/dt = -\ k_{in}(B_0-B_L), \qquad (2\text{-}17)$$

which may be solved simultaneously with the rate equation (2-1) by standard computerized numerical integration methods (e.g., Yeakley et al., 1980).

Applications and Discussion

If the chosen binding assay can be performed rapidly then the association rate constant may be determined conveniently by measuring B_L as a function of t for a relatively short period of time (during which dissociation may be neglected) following the mixing of ligand with the receptor preparation. If t is small then dissociation may be neglected and equation (2-1) becomes simply

$$dB_L/dt = k_a(S_L-B_L)(B_0-B_L), \tag{2-18}$$

which, upon integration with the initial condition that $B_L=0$ at $t=0$, gives

$$k_a t = [1/(S_L-B_0)] \ln ([B_0(B_L-S_L)]/[S_L(B_L-B_0)]). \tag{2-19}$$

It follows immediately that

$$k_a t\ (S_L-B_0) = \ln (B_0/S_L) + \ln [(S_L-B_L)/(B_0-B_L)]. \tag{2-20}$$

It is apparent that at short times a plot of $\ln [(S_L-B_L)/(B_0-B_L)]$ as a function of time will be a line with slope $k_a\ (S_L-B_0)$. The ordinate of this plot is more easily remembered as ln [(free ligand)/(free receptor)]. This method for the determination of k_a obviously requires both knowledge of S_L and an estimate of B_0 derived from an equilibrium experiment performed with the same receptor preparation.

It is also possible to determine the association constant k_a by measuring B_L following brief incubations of constant (short) duration

conducted at different ligand concentrations. These values of B_L, when divided by the incubation time, are taken as estimates of the initial rate of increase of binding (dB_L/dt), which is plotted on the ordinate as a function of the total ligand S_L. To analyze this experimental strategy we note that since $B_L = 0$ at $t = 0$, equation (2-1) reveals that the initial rate of appearance of bound complex is given simply by

$$dB_L/dt \text{ (at } t=0) = B_0 k_a S_L. \qquad (2\text{-}21)$$

Thus, the plot of dB_L/dt (initial) as a function of S_L is a line possessing slope $B_0 k_a$. In fact, the observed curvature of such a plot (a decrease in slope at high values of S_L) has even been offered as possible evidence that the glucocorticoid receptor binding process may involve multiple steps and unobserved transient intermediate states (Pratt, Kaine and Pratt, 1975). (The most probable explanation of this anomaly is that the constant incubation time was too long to provide a reliable estimate of the initial $dB_L dt$ at the high ligand concentrations.) The two aforementioned experimental designs may be combined, of course, by accumulating the early regions of temporal binding curves generated at different ligand concentrations. The individual binding curves will generate independent measurement of k_a that should not be correlated with the ligand concentration S_L. Information (in the form of the derived values of k_a) from the individual binding curves may also be merged by plotting $(1/B_0)dB_L/dt$ (initial) = $k_a S_L$ as the ordinate vs. S_L as the abscissa for each binding curve. The plot should pass through the origin, and the resulting slope provides the merged estimate of k_a.

The dissociation rate constant k_d is determined by simply measuring the bound ligand B_L as a function of time following some manipulation that prevents any further association (or reassociation) of labeled ligand with the receptors. Ordinarily this is accomplished by adsorbing the free labeled steroid onto activated charcoal, followed by either dilution of the preparation to further reduce the concentration of free steroid to a negligible level or by the addition of a high concentration of unlabeled steroid to dilute the specific activity of any remaining labeled free steriod (and to dilute the labeled steroid that is released into the free pool during the course of dissociation). Thus, since the association of labeled ligand is blocked, equation (2-1) becomes

$$dB_L/dt = - k_d B_L, \tag{2-22}$$

where B_L now represents only labeled bound ligand. Upon integration we obtain the familiar first order dissociation relations

$$\ln[B_L/(B_L)_0] = - k_d t \tag{2-23}$$

and finally

$$B_L = (B_L)_0 \exp(-k_d t), \tag{2-24}$$

where $(B_L)_0$ is the initial bound concentration at the beginning of the dissociation period. The rate constant k_d is simply the slope of the plot of $\ln[B_L/(B_L)_0]$ as a function of t. The dissociation rate is also frequently reported as the half-life ($t_{1/2}$) of the bound complex:

$$t_{1/2} = (\ln 2)/k_d. \qquad (2\text{-}25)$$

Depending on the experimental design, a measurement of the dissociation rate constant may be performed under conditions of decreasing, constant, or increasing receptor occupancy (B_L/B_0). If no unlabeled steroid is added, the occupancy obviously will decline. (However, if unoccupied receptors are inactivated or degraded at a rate comparable to the dissociation rate, then occupancy will not decline as rapidly as B_L itself.) If the receptors are saturated at t=0, then addition of a large amount of unlabeled ligand will maintain the high level of binding. If the receptors are not initially near saturation, then the addition of unlabeled steroid will lead to a condition of rapidly increasing occupancy. If necessary, the concentration of unlabeled ligand may be chosen to maintain a desired intermediate level of occupancy. If the dissociation kinetics are biphasic or complex under conditions of constant occupancy or saturation, then the possibility of several classes of binding sites must be considered (e.g., Weichman and Notides, 1979). If the kinetics are complex under conditions of declining occupancy, then the possibility of cooperativity must be considered; the experiment should then be repeated at high or constant occupancy to see if the apparent cooperativity is really a reflection of heterogeneity (e.g., DeMeyts, Roth, Neville, Gavin and Lesniak, 1973).

If excess unlabeled steroid is not added to the preparation at the beginning of the dissociation period, the possible reassociation of newly-dissociated labeled ligand or the association of the small amount of residual labeled steroid not removed initially may reduce the

measured apparent dissociation constant. The magnitude of this reduction may be estimated approximately by using the solution to the rate equation to monitor the relaxation of B_L to the new, low-but-nonzero value of P (equilibrium value of B_L) in the following way. First, assume for k_d the (underestimated) value that has been measured experimentally; call this value k_d(apparent). A previously estimated value of k_a or K_{dL} must also be assumed; if a value of k_a is assumed, then K_{dL} is taken to be k_d(apparent)/k_a. Measured values of S_L, B_0, and $(B_L)_0$ are also available. Now equation (2-12) is used with these values of the independent variables to calculate B_L for the same values of t at which actual measurements of B_L have been made. These calculated values of $B_L(t)$ are then used to derive, from the slope of a plot of t vs. ln [calculated $B_L(t)/(B_L)_0$], another underestimated dissociation rate constant, called k_d'. Next assume simply that the amount by which k_d' underestimates k_d(apparent) is equal to the amount by which k_d(apparent) underestimates the "true" value of k_d that is sought. Thus

$$\text{"true" } k_d - k_d(\text{apparent}) \simeq k_d(\text{apparent}) - k_d', \tag{2-26}$$

and finally

$$\text{"true" } k_d \simeq 2\, k_d(\text{apparent}) - k_d'. \tag{2-27}$$

This rough estimate of the correction is reasonable when S_L is small.

The solution to the rate equation may also be used to predict the error in a determination of K_{dL} derived from a "nonequilibrium" isotherm generated by measurements performed before equilibrium has been attained in the incubation vessels containing the lower concentrations of S_L.

(Estimates of the rate constants and B_0 are required, of course, in order to perform this simulation.) A predicted value of B_L is calculated for each experimental value of S_L (at the constant value of t used for the incubation) using equation (2-13), and the resulting values are used to construct the predicted (curved) Scatchard plot resulting from the inadequate incubation time. A line is fitted to the points by the same technique that is used to fit the experimental points (e.g., linear regression), and a value of K_{dL} is derived from the resulting "slope" (e.g., right inset to fig. 4-10). The entire procedure may be iterated for different values of t in order to simulate the approach of the apparent, measured K_{dL} to its "true" equilibrium value as the incubation time is prolonged. It is instructive to examine the dependence of the relative error function upon S_L; when combined with equation (2-13), equation (2-14) becomes

$$\varepsilon = (Q-P)\exp(-ft)/[Q-P\exp(-ft)], \qquad (2\text{-}28)$$

which is plotted as a function of S_L in fig. 4-10 (left inset) for several different incubation times using one specific set of the other independent variables. It is apparent that ε rises rapidly to a maximum and then declines slightly as S_L is decreased from a high concentration. Thus, as S_L is decreased below the transition zone, ε becomes essentially constant at a value given by equation (2-29) below, which is simply the limiting value of ε as S_L declines:

$$\lim \varepsilon \text{ (as } S_L \text{ is decreased)} = \exp(-ft) = \exp\left[-k_a t(K_{dL}+B_0)\right]. \qquad (2\text{-}29)$$

Thus, if a time and receptor concentration B_0 are chosen such that equation (2-29) is reduced to an acceptable level, then very low values of S_L may be used to generate an isotherm. (More rigorously, one may find by setting equal to 0 the partial derivative of equation (2-28) with respect to S_L, the exact nonzero value of S_L that maximizes ε; then a time may be chosen that reduces this maximum relative error to an acceptable level.)

Predicting the effect of the addition of a competing, cold ligand (C) on the rate of approach to equilibrium of B_L is a more complex problem; this calculation requires the numerical integration of the two simultaneous rate equations

$$dB_L/dt = k_a (B_0-B_L-B_C)(S_L-B_L) - k_d B_L \qquad (2\text{-}30)$$

and

$$dB_C/dt = k_{aC} (B_0-B_L-B_C)(S_C-B_C) - k_{dC} B_C, \qquad (2\text{-}31)$$

where the subscript C refers to the competing ligand. Several qualitative inferences can be drawn, however, from the fact that the only effect of the competitor C is to decrease the free receptor concentration in equation (2-30). Consider now the relative error ε as a function of B_0 rather than of S_L; since S_L and B_0 appear symmetrically in equation (2-28), the left inset to fig. 4-10 also portrays the shape of the plot of ε considered as a function of B_0 (with S_L now held fixed). If S_L is sufficiently large, then the position of B_0 will be to the left of the maximum value of ε on the plot; if S_L is small enough,

however, the position of the same value of B_0 will now be to the right of the ε maximum value. Thus, depending on the relations among S_L, B_0 and K_{dL}, the effect of the reduction of free receptor concentration by the competing ligand can be either to increase, decrease, or leave essentially unchanged the value of ε at a given time. Under the right conditions a competitor may easily increase ε from near 0 to the maximum value consistent with the given values of S_L and t. (The rate constants for ligand C and its concentration will, of course, affect ε by determining how fast the competitor depletes the free receptor concentration.) A very rough estimate of the effect of a high-affinity competing ligand on the time required for B_L to approach equilibrium may be obtained simply by assuming that the competitor is identical to the labeled ligand. In this simple case S_L becomes the sum of the labeled and competing ligands, and equation (2-28) predicts ε for the total bound ligand ($B_L + B_C$) for different times. Clearly this value of the relative error also applies to B_L, since for this special case

$$B_L = S_L\ (B_L+B_C)/(S_L+S_C). \tag{2-32}$$

In order to facilitate estimation of the times required for the approach to equilibrium under a variety of conditions, we present in table 2-1 a short summary of solutions to the rate equation (2-1) for several useful combinations of the variables B_0, S_L, and K_{dL} (i.e., k_d/k_a) for the case of a single ligand and binding site. The initial condition is simply $(B_L)_0 = 0$, and the solution is presented as a table of pairs of numbers representing the number of hours required for B_L to attain 80% and 95%, respectively, of the equilibrium value. These

Table 2-1. Number of hours required for the concentration of specifically-bound ligand (B_L) to reach 80% and 95% (upper and lower number in each pair, respectively) of true equilibrium for a noncooperative, single site binding system in which the second-order rate constant (k_a) equals $10^5 M^{-1} min^{-1}$. For other values of k_a multiply the tabulated values by 10^5 and divide the product by k_a. B_0 is the total number of binding sites, S_L is the total ligand concentration and K_{dL} is the equilibrium constant.

- Log S_L	-Log B_o = 7	8	9	10	11	7	8	9	10	11	7	8	9	10	11	7	8	9	10	11
	- Log K_{dL} = 8					- Log K_{dL} = 9					- Log K_{dL} = 10					- Log K_{dL} = 11				
6	0.28	0.27	0.27	0.27	0.27	0.28	0.27	0.27	0.27	0.27	0.28	0.27	0.27	0.27	0.27	0.28	0.27	0.27	0.27	0.27
	0.53	0.50	0.49	0.49	0.49	0.54	0.50	0.50	0.50	0.50	0.54	0.50	0.50	0.50	0.50	0.54	0.50	0.50	0.50	0.50
6.5	0.96	0.83	0.82	0.82	0.82	1.01	0.86	0.85	0.85	0.85	1.02	0.86	0.85	0.85	0.85	1.02	0.86	0.85	0.85	0.85
	1.90	1.56	1.53	1.53	1.53	2.02	1.61	1.58	1.57	1.57	2.03	1.61	1.58	1.58	1.58	2.03	1.61	1.58	1.58	1.58
7	2.74	2.52	2.45	2.44	2.44	4.54	2.79	2.67	2.66	2.66	5.78	2.82	2.69	2.68	2.68	6.35	2.83	2.70	2.68	2.68
	5.96	4.76	4.56	4.54	4.54	12.41	5.30	4.98	4.95	4.94	20.34	5.36	5.02	4.99	4.99	26.62	5.36	5.03	5.00	4.99
7.5	2.70	6.56	6.47	6.45	6.44	3.15	9.55	8.34	8.23	8.22	3.21	10.09	8.59	8.47	8.46	3.21	10.15	8.62	8.49	8.48
	5.29	12.61	12.07	12.00	12.00	6.29	18.97	15.61	15.33	15.31	8.42	20.19	16.08	15.77	15.74	6.43	20.32	16.13	15.82	15.79
8	2.52	11.07	13.24	13.40	13.41	2.79	27.44	25.21	24.47	24.39	2.82	45.37	27.92	26.69	26.57	2.83	57.77	28.23	26.93	26.81
	4.76	21.22	24.74	24.94	24.96	5.30	59.62	47.65	45.61	45.41	5.36	124.1	52.95	49.76	49.47	5.36	203.5	53.56	50.22	49.91
8.5	2.46	12.79	19.41	20.28	20.37	2.70	26.96	65.61	64.66	64.47	2.72	31.48	95.51	83.39	82.34	2.73	32.05	100.9	85.91	84.69
	4.61	24.12	36.23	37.76	37.92	5.05	52.90	126.1	120.7	120.0	5.10	62.86	189.7	156.1	153.4	5.10	64.16	201.9	160.8	157.7
9	2.45	13.24	22.59	24.20	24.37	2.67	25.21	110.7	132.4	134.0	2.69	27.92	274.4	252.1	244.7	2.70	28.23	453.7	279.2	266.9
	4.56	24.74	42.10	45.04	45.35	4.98	47.65	212.2	247.4	249.4	5.02	52.95	596.2	476.5	456.1	5.03	53.56	1241	529.5	497.6
9.5	2.44	13.36	23.79	25.76	25.98	2.66	24.64	127.9	194.1	202.8	2.68	26.97	296.6	656.1	646.6	2.69	27.23	314.8	955.1	833.9
	4.55	24.90	44.31	47.96	48.35	4.95	46.09	241.2	362.3	377.6	5.00	50.49	529.0	1261	1207	5.00	50.98	628.6	1897	1561
10	2.44	13.40	24.20	26.30	26.53	2.66	24.47	132.4	225.9	242.0	2.68	26.69	252.1	1107	1324	2.68	26.93	279.2	2744	2521
	4.54	24.94	45.04	48.96	49.39	4.95	45.61	247.4	221.0	450.4	4.99	49.76	476.5	2122	2474	5.00	50.22	529.5	5962	4765
10.5	2.44	13.41	24.33	26.48	26.71	2.66	24.41	133.6	237.9	257.6	2.68	26.60	246.4	1279	1941	2.68	26.84	269.7	2696	6561
	4.54	24.96	45.28	49.28	45.72	4.94	45.46	249.0	443.1	479.6	4.99	49.54	460.9	2412	3623	4.99	49.99	505.0	2590	12612
11	2.44	13.41	24.37	26.53	26.77	2.66	24.39	134.0	242.0	263.0	2.68	26.57	245.0	1324	2259	2.68	26.81	267.0	2521	11071
	4.54	24.96	45.35	49.39	49.83	4.94	45.41	249.4	450.4	490.0	4.99	49.47	456.1	2474	4210	4.99	49.91	498.0	4765	21217
11.5	2.44	13.41	24.38	26.55	26.79	2.66	24.39	134.1	243.3	264.8	2.68	26.56	244.1	1336	2379	2.68	26.80	266.0	2464	12793
	4.54	24.96	45.38	49.42	49.86	4.94	45.40	249.6	452.8	492.8	4.99	49.44	454.6	2490	4431	4.99	49.89	494.4	4609	24116
12	2.44	13.41	24.38	26.56	26.79	2.66	24.39	134.1	243.7	265.3	2.68	26.56	243.9	1340	2420	2.68	26.80	265.7	2447	13235
	4.54	24.96	45.39	49.43	49.87	4.94	45.39	249.6	453.5	493.9	4.99	49.44	454.1	2494	4504	4.99	49.88	494.7	4561	24743

Table 2-2. Fraction of specific binding sites occupied and the fraction of the total ligand involved in that binding at equilibrium for a noncooperative, single site binding system. Tabulated data are expressed as percentages with B_L/B_0 (x100) presented as the upper and B_L/S_L (x100) as the lower number in each pair. B_L is the concentration of specifically-bound ligand, B_0 is the total number of binding sites, S_L is the total ligand concentration and K_{dL} is the equilibrium constant.

- Log S_L	- Log B_o = 7	8	9	10	11
	-Log K_{dL} = 8				
6	98.9 9.9	99.0 1.0	99.0 0.1	99.0 <0.1	99.0 <0.1
6.5	95.7 30.2	96.8 3.1	96.9 0.3	96.9 <0.1	96.9 <0.1
7	73.0 73.0	90.1 9.0	90.8 0.9	90.9 0.1	90.9 <0.1
7.5	27.8 87.8	71.0 22.5	75.5 2.4	75.9 0.2	76.0 <0.1
8	9.0 90.1	38.2 38.2	48.8 4.9	49.9 0.5	50.0 <0.1
8.5	2.9 90.7	14.6 46.1	22.7 7.2	23.9 0.8	24.0 0.1
9	0.9 90.8	4.9 48.8	8.4 8.4	9.0 0.9	9.1 0.1
9.5	0.3 90.9	1.6 49.6	2.8 8.9	3.0 1.0	3.1 0.1
10	0.1 90.0	0.5 49.9	0.9 9.0	1.0 1.0	1.0 0.1
10.5	<0.1 90.9	0.2 50.0	0.3 9.1	0.3 1.0	0.3 0.1
11	<0.1 90.9	<0.1 50.0	0.1 9.1	0.1 1.0	0.1 0.1
11.5	<0.1 99.9	<0.1 50.0	<0.1 9.1	<0.1 1.0	<0.1 0.1
12	<0.1 90.9	<0.1 50.0	<0.1 9.1	<0.1 1.0	<0.1 0.1
	-Log K_{dL} = 9				
6	99.9 10.0	99.9 1.0	99.9 0.1	99.9 <0.1	99.9 <0.1
6.5	99.5 31.5	99.7 3.2	99.7 0.3	99.7 <0.1	99.7 <0.1
7	90.5 90.5	98.9 9.9	99.0 1.0	99.0 0.1	99.0 <0.1
7.5	31.2 98.6	95.7 30.2	96.8 3.1	96.9 0.3	96.9 <0.1
8	9.9 98.9	73.0 73.0	90.1 9.0	90.8 0.9	90.9 0.1
8.5	3.1 99.9	27.8 87.8	71.0 22.5	75.5 2.4	75.9 0.2
9	1.0 99.0	9.0 90.1	38.2 38.2	48.8 4.9	49.9 0.5
9.5	0.3 99.0	2.9 90.7	14.6 46.1	22.7 7.2	23.9 0.8
10	0.1 99.0	0.9 90.9	4.9 48.8	8.4 8.4	9.0 0.9
10.5	<0.1 99.0	0.3 90.9	1.6 49.6	2.8 8.9	3.0 1.0
11	<0.1 99.0	0.1 90.9	0.5 49.9	0.9 9.0	1.0 1.0
11.5	<0.1 99.0	<0.1 90.9	0.2 50.0	0.3 9.1	0.3 1.0
12	<0.1 99.0	<0.1 90.9	<0.1 50.0	0.1 9.1	0.1 1.0
	-Log K_{dL} = 10				
6	>99.9 10.0	>99.9 1.0	>99.9 0.1	>99.9 <0.1	>99.9 <0.1
6.5	>99.9 31.6	>99.9 3.2	>99.9 0.3	>99.9 <0.1	>99.9 <0.1
7	96.9 96.9	99.9 10.0	99.9 1.0	99.9 0.1	99.9 <0.1
7.5	31.6 99.8	99.5 31.5	99.7 3.2	99.7 0.3	99.7 0.1
8	10.0 99.9	90.5 90.5	98.9 9.9	99.0 1.0	99.0 0.1
8.5	3.2 99.9	31.2 98.6	95.7 30.2	96.8 3.1	96.9 0.3
9	1.0 99.9	9.9 98.9	73.0 73.0	90.1 9.0	90.8 0.9
9.5	0.3 99.9	3.1 99.0	27.8 87.8	71.0 22.5	75.5 2.4
10	0.1 99.9	1.0 99.0	9.0 90.1	38.2 38.2	48.8 4.9
10.5	<0.1 99.9	0.3 99.0	2.9 90.7	14.6 46.1	22.7 7.2
11	<0.1 99.9	0.1 99.0	0.9 90.8	4.8 48.8	8.4 8.4
11.5	<0.1 99.9	<0.1 99.0	0.3 90.9	1.6 49.6	2.8 8.9
12	<0.1 99.9	<0.1 99.0	0.1 90.9	0.5 49.9	0.9 9.0
	-Log K_{dL} = 11				
6	>99.9 10.0	>99.9 1.0	>99.9 0.1	>99.9 <0.1	>99.9 <0.1
6.5	>99.9 31.6	>99.9 3.2	>99.9 0.3	>99.9 <0.1	>99.9 <0.1
7	99.0 99.9	99.9 10.0	>99.9 1.0	>99.9 0.1	>99.9 <0.1
7.5	31.6 99.9	99.9 31.6	99.9 3.2	>99.9 0.3	>99.9 0.1
8	10.0 >99.9	96.9 96.9	99.9 10.0	99.9 1.0	99.9 0.1
8.5	3.2 >99.9	31.6 99.8	99.5 31.5	99.7 3.2	99.7 0.3
9	1.0 >99.9	10.0 99.9	90.5 90.5	98.9 9.9	99.0 1.0
9.5	0.3 >99.9	3.2 99.9	31.2 98.6	95.7 30.2	96.8 3.1
10	0.1 >99.9	1.0 99.9	9.9 98.9	73.0 73.0	90.1 9.0
10.5	<0.1 >99.9	0.3 99.9	3.1 99.0	27.8 87.8	71.0 22.5
11	<0.1 >99.9	0.1 99.9	1.0 99.0	9.0 90.1	38.2 38.2
11.5	<0.1 >99.9	<0.1 99.9	0.3 99.0	2.9 90.7	14.6 46.1
12	<0.1 >99.9	<0.1 99.9	0.1 99.0	0.9 90.8	4.9 48.8

durations were calculated for $k_a = 10^5\ M^{-1}\ min^{-1}$. For other values of k_a simply multiply the tabulated values by 10^5 and divide the resulting product by k_a. Table 2-2 presents estimates of the fractional occupancy of the binding sites (B_L/B_0) and the fraction of the ligand bound at equilibrium (P/S_L) under the same hypothetical conditions as in table 2-1. (These percentages apply to all values of k_a.)

CHAPTER III

LINEARIZATION OF THE TWO LIGAND-SINGLE BINDING SITE SCATCHARD PLOT AND "ED_{50}" COMPETITION DISPLACEMENT PLOT: APPLICATION TO THE SIMPLIFIED GRAPHICAL DETERMINATION OF EQUILIBRIUM CONSTANTS

Introduction

The affinity of a ligand for a particular class of binding sites is measured frequently by constructing an isotherm describing the binding of an available labeled ligand having specificity for the same set of binding sites, first in the absence and then in the presence of a fixed total concentration of the unlabeled competitive inhibitor whose affinity constant is to be estimated (e.g., Ginsburg, MacLusky, Morris and Thomas, 1977; Katzenellenbogen, Katzenellenbogen, Ferguson and Krauthammer, 1978; Kono, 1975; for review of competition experimental designs see Rodbard, 1973). The data resulting from such an experiment are often analyzed by making the approximation that the concentration of free competitive inhibitor is equal to the total concentration present and therefore constant over the entire range of labeled ligand concentration, since the effect of a constant concentration of free competitive inhibitor on a Scatchard plot (Scatchard, 1949) describing the binding of a labeled ligand to a single class of noncooperative binding sites is solely to decrease the slope of the plot by a factor which, in combination with the concentration of free competitive inhibitor, yields immediately the equilibrium constant of the inhibitor

(Cantor and Schimmel, 1980). Thus, if the above approximation is valid, then the affinity of the competitor results directly from the slopes of the two straight lines and the total concentration of the inhibitor. However, if the competitive inhibitor is not present in great excess over the total concentration of binding sites, then the above approximation will not be valid, the resulting Scatchard plot will be curved (Feldman, 1972), and the error in the derived equilibrium constant caused by making the approximation may be substantial. In the present communication we suggest a simple procedure for eliminating this error by linearizing the curved Scatchard plot resulting from this experimental design.

The very popular competition displacement experimental design (known as the "ED_{50}" method) also generates data that are somewhat difficult to analyze in the laboratory without the aid of computerized nonlinear regression techniques. Within this design one measures the fraction of initially bound labeled ligand remaining bound at equilibrium in the presence of increasing concentrations of the unlabeled competitive inhibitor whose affinity is to be measured (e.g., Abrass & Scarpace, 1981; Lindenbaum & Chatterton, 1981; for review, see Rodbard, 1973). The total concentration of inhibitor that displaces half the initially bound labeled ligand ("ED_{50}") is determined, and one then attempts to relate this inhibitor concentration to its actual affinity for the binding sites. This design presents two major problems: the curvature of the displacement plot makes the precise determination of ED_{50} difficult, and the ED_{50} itself is often quite different from, and difficult to relate to, the actual equilibrium dissociation constant of the competing ligand. In many situations the

curvature of the competition displacement plot cannot be eliminated simply by performing a "logit-log" transformation (e.g., De Lean, Munson and Rodbard, 1978) of the data. Furthermore, the use of approximations (e.g., the Cheng-Prusoff (1973) formula) to relate the estimated ED_{50} to the equilibrium dissociation constant of the competitor is often inappropriate and can lead to substantial additional error.

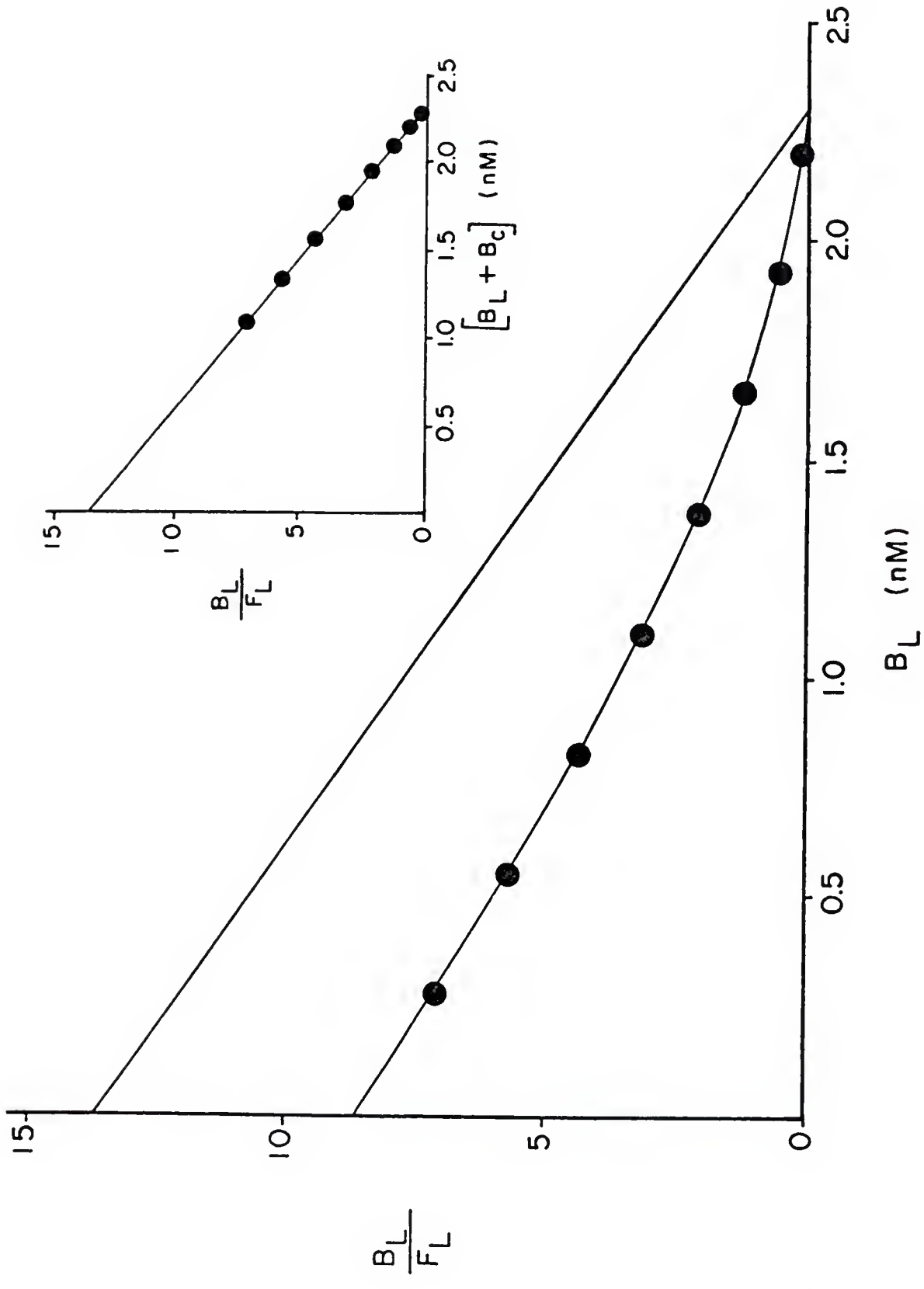
We now suggest that if the initial receptor occupancy is not too high this experimental design can also be analyzed without approximation by the simple procedure (to be described) that we recommend for linearization of the curved, two-ligand Scatchard plot obtained in the presence of a fixed concentration of competitive inhibitor. With this method the equilibrium dissociation constant is obtained directly, obviating calculation of the ED_{50} value itself. Furthermore, a more nearly exact approximation that can be used to relate ED_{50} estimated from an approximately linear logit-log plot of the competition displacement data to the actual affinity of the competitive inhibitor for the binding sites is presented. This method is particularly useful when the initial receptor occupancy is rather high.

Theory and Application

The nomenclature for the two ligand-one binding site problem will be as follows: B_L and B_C are, respectively, the concentrations of specifically bound labeled ligand L and competitive inhibitor C. The total concentration of binding sites is B_0. The equilibrium dissociation constants for the binding of the labeled ligand and competitor are, respectively, K_{dL} and K_{dC}; and S_L and S_C are the total concentrations of these ligands. The free (unbound) concentrations of

Fig. 3-1. Theoretical Scatchard plots for a single ligand and for two ligands in competition for one class of noncooperative binding sites. Inset: linearization of the curved Scatchard plot by plotting the total concentration of occupied binding sites (B_L+B_C) on the abscissa.

The parameters used to generate the plots describe the binding of estradiol (E_2) and estriol (E_3) to the nonactivated calf uterine estrogen receptor and were taken from Weichman and Notides (1980). Upper straight line: E_2 alone, equilibrium dissociation constant $K_{dL} = 1.7 \times 10^{-10}$M; lower curved plot: E_2 in the presence of 1 nM estriol (E_3, equilibrium constant $K_{dC} = 2.6 \times 10^{-10}$M). The concentration of binding sites (B_0) is 2.3 nM. The curved Scatchard plot for the purely competitive two ligand situation is a hyperbola whose properties have been described (Feldman, 1972). The actual points on the curve are purely hypothetical and were placed on the plot to indicate how specific binding values map into the linearized coordinate systems shown in the inset and in Fig. 3-2 and discussed in the text. The slope of the transformed plot remains the same ($-1/K_{dL}$) as that of the original linear plot generated by the single labeled ligand (E_2), as does the intercept on the x-axis (B_0).


15
10
5
0
0.5
1.0
1.5
2.0
2.5
$\frac{B_L}{F_L}$
B_L (nM)
$[B_L + B_c]$ (nM)

the ligands are F_L and F_C. In order to discuss the "ED_{50}" experimental design we let $(B_L)_0$ and $(F_L)_0$ represent the initial values of B_L and F_L when S_C=0. ED_{50} is the value of S_C when B_L has been reduced by half (i.e., when $B = (B_L)_0/2$), and we let F_{C50} and F_{L50} represent the corresponding values of F_C and F_L (i.e., when $S_C = ED_{50}$).

As the first illustrative example we have used the equilibrium dissociation constants that describe the binding of estradiol (E_2) and estriol (E_3) to the nonactivated calf uterine cytosol estrogen receptor (Weichman and Notides, 1980) in order to generate the purely hypothetical Scatchard plots (Fig. 3-1) of theoretical data that would be observed for the binding of labeled E_2, first in the absence and then in the presence of S_C = 1 nM "cold" E_3. (The experimental design under discussion here was not employed by Weichman and Notides (1980); we have merely used the affinity constants, which were measured and reported appropriately by these authors.) The concentration of binding sites B_0 = 2.3 nM is also taken from Weichman and Notides (1980); the equilibrium constants are $K_{dL} = 1.7 \times 10^{-10}$M (for E_2) and $K_{dC} = 2.6 \times 10^{-10}$M (for E_3). The linear Scatchard plot (for the case $S_C = 0$) is, of course, described by the standard equation (e.g., Rodbard, 1973; Scatchard, 1949):

$$B_L/F_L = - (B_L - B_0)/K_{dL} \qquad (3\text{-}1)$$

and thus has slope $(-1/K_{dL})$ and intercept B_0 on the x-axis (Fig. 3-1). The lower, curvilinear Scatchard plot (Fig. 3-1) for the purely competitive two ligand situation is a hyperbola whose geometric

properties have been described by Feldman (1972) and whose equation is (Feldman, 1972; Rodbard and Feldman, 1975)

$$B_L/F_L = K_{dC}[-1-(B_L+S_C-B_0)/K_{dC}+[(1-[B_L+S_C-B_0]/K_{dC})^2 + 4S_C/K_{dC}]^{1/2}]/2K_{dL}. \quad (3\text{-}2)$$

This hyperbolic Scatchard plot has one asymptote with slope $(-1/K_{dL})$ parallel to the linear Scarchard plot and an x-intercept (B_0-S_C), and another horizontal asymptote below the x-axis at $B_L/F_L = (-K_{dC}/K_{dL})$. The actual points on the curve are completely hypothetical and have been calculated and placed on the plot at equally spaced intervals of B_L (0.275 nM) in order to indicate how specific binding values map into the modified coordinate systems to be discussed.

We now calculate the error in the derived value of K_{dC} resulting from the assumption that the concentration of free inhibitor F_C is constant in this experiment and therefore that the expression (derived from simple Michaelis-Menten kinetics for the case of pure competitive inhibition) sometimes called the Edsall-Wyman equation (Cantor and Schimmel, 1980) is valid when applied to some estimate of the "slope" of this (actually curved) plot. The Edsall-Wyman equation can be rearranged to the convenient "Scatchard" form given by (cf. equation 3-1)

$$B_L/F_L = - (B_L-B_0)/K_{dL}(1+F_C/K_{dC}). \quad (3\text{-}3)$$

Thus, it predicts simply that the slope of the Scatchard plot will be reduced by a factor of $1/(1+F_C/K_{dC})$ in the presence of a constant concentration of free inhibitor F_C. We now substitute the total concentration S_C for the (actually variable) free concentration F_C. Thus, the estimated value of K_{dC} derived from the use of this approximation (which we call $K_{dC}(app)$ will be given by

$$K_{dC}(app) = [\text{reduced slope}/(\text{original slope}-\text{reduced slope})]S_C. \tag{3-4}$$

Thus, the percentage error (E) will be given by

$$E/100 = [K_{dC}(app)/K_{dC}]-1 \tag{3-5}$$

and hence, from above,

$$E/100 = (-K_{dL}S_C)(\text{reduced slope})/K_{dC}[1+K_{dL}(\text{reduced slope})]-1. \tag{3-6}$$

For the "reduced slope" we substitute the derivative of the equation of the curvilinear Scatchard plot, $d(B_L/F_L)/dB_L$, obtained directly from equation (3-2) above. Thus, if the error is estimated by using as the "reduced slope" the slope of the tangent to the curve at the abscissa $B_L = B_0/2$, then the error in the derived equilibrium constant is given explicitly by

$$E/100 = (K_{dC}[S_C-K_{dC}]Y-X[S_C+K_{dC}])/(K_{dC}{}^2Y+K_{dC}X), \tag{3-7}$$

where $X = S_C - K_{dC} - (B_0/2)$ and

$$Y = ([1-(2S_C-B_0)/2K_{dC}]^2+4S_C/K_{dC})^{1/2}.$$

Thus, the error is independent of K_{dL} and vanishes under the ideal conditions approached when $K_{dC} >> B_0$ and $S_C \simeq K_{dC}$. In the hypothetical example involving estradiol (E_2) and estriol (E_3) the error resulting from the use of this approximate method is large: the derived K_{dC} (calculated from the slope obtained from a linear regression on the eight hypothetical points shown on the curvilinear Scatchard plot in Fig. 3-1) for E_3 is 1.6×10^{-9}M, whereas the actual value used to generate the curve is 2.6×10^{-10}M, a 5-fold discrepancy. In fact, a significant and variable fraction of the competitor E_3 is obviously bound to the receptors in this hypothetical experiment.

The error discussed above may be avoided by analyzing the data from the same experimental design in a slightly different manner. At equilibrium the simple mass action rate equations for the binding of the two ligands may be written as

$$(B_0-B_L-B_C)F_L = K_{dL}B_L \text{ and} \qquad (3\text{-}8)$$

$$(B_0-B_L-B_C)F_C = K_{dC}B_C, \qquad (3\text{-}9)$$

which suggest immediately the "linearized Scatchard" forms given by

$$B_L/F_L = -[(B_L+B_C)-B_0]/K_{dL} \text{ and} \qquad (3\text{-}10)$$

$$B_C/F_C = -[(B_L+B_C)-B_0]/K_{dC}. \qquad (3\text{-}11)$$

Thus, if B_L/F_L or B_C/F_C on the ordinate are plotted against $(B_L + B_C)$ on the abscissa, then a linear "Scatchard-like plot with slope $(-1/K_{dL})$ or $(-1/K_{dC})$ and x-intercept B_0 results (insets to Figs. 3-1 and 3-2). Furthermore, since B_0 (as well as K_{dL}) is derived from an initial Scatchard plot constructed in the absence of the competitor, K_{dC} may be measured simply by plotting $[B_0-B_L-B_C$, (free binding sites)] on the abscissa against B_C/F_C on the ordinate, as suggested by equation (3-9). The resulting plot (Fig. 3-2) must pass through the origin and possess slope $(1/K_{dC})$. This is the analysis we recommend for the determination of K_{dC} by this experimental method.

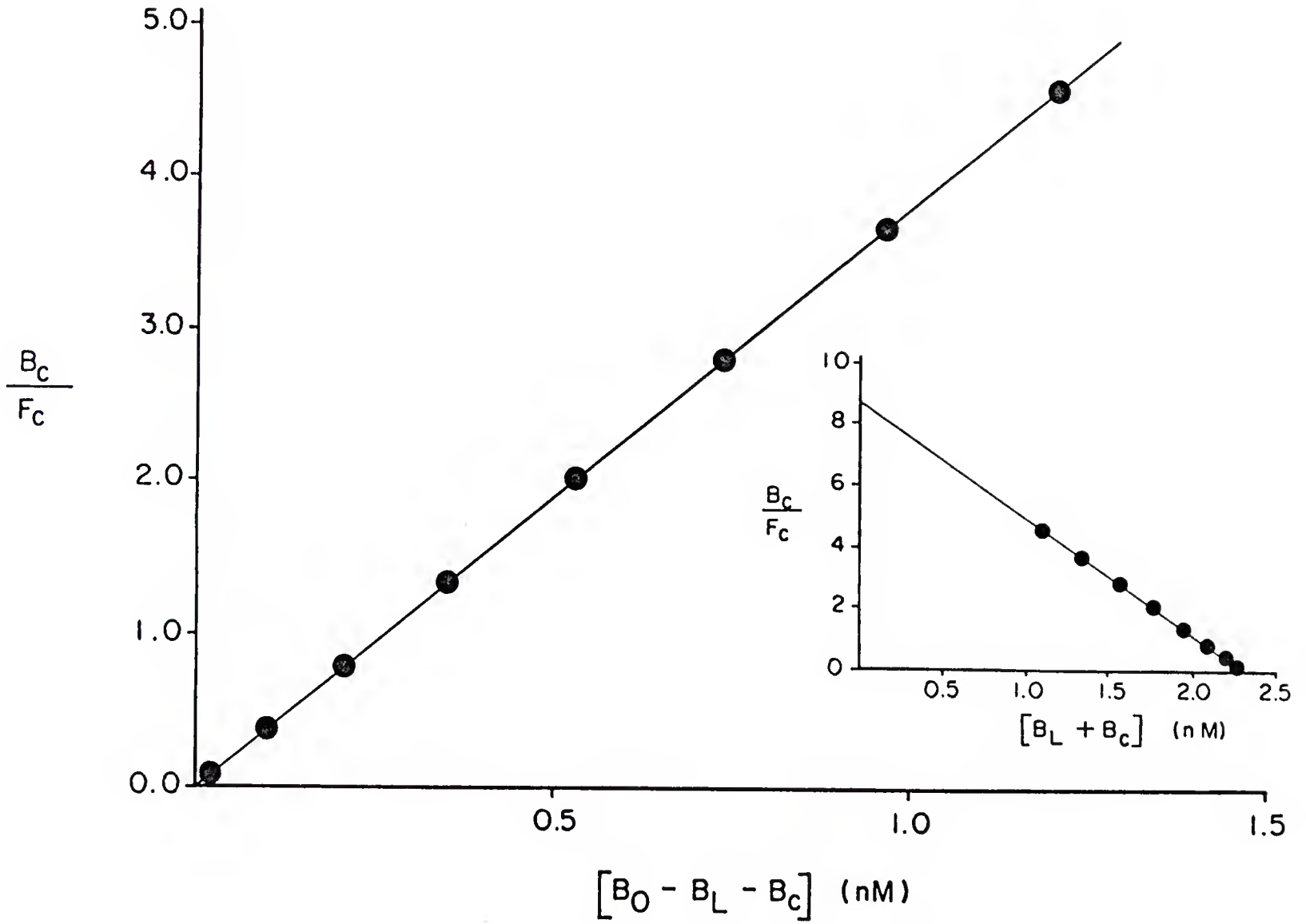
The implementation of this analysis is scarcely more difficult than the method that we have criticized: the only additional requirement is the calculation of B_C and F_C for each data point. We note that B_0 and K_{dL} have been determined already from an initial Scatchard plot. Equation (3-8) is now rearranged to give

$$B_0-B_L-B_C = K_{dL}B_L/F_L. \qquad (3\text{-}12)$$

Thus, the abscissa of the recommended plot is determined directly from equation (3-12), as is the value of $B_C = [B_0-B_L-(K_{dL}B_L/F_L)]$. Since S_C is known, $F_C \simeq S_C-B_C$. (Note that nonspecific binding of the "cold" competitive ligand must still be neglected, unless it can be estimated from other experiments, e.g. Katzenellenbogen et al., 1978). The best-fitting straight line passing through the origin is then fit to the points as shown (Fig. 3-2), and the reciprocal of its slope is then

Fig. 3-2. Linearization of the curved Scatchard plot shown in Fig. 3-1.

The same hypothetical data points presented in Fig. 3-1 are shown plotted in the coordinate system recommended for the determination of K_{dC}. The ordinate (B_C/F_C) now refers explicitly to the concentrations of bound and free competitor (E_3), and the concentration of free binding sites $(B_0-B_L-B_C)$ is plotted on the abscissa. The plot must pass through the origin, and the slope $(1/K_{dC})$ is the reciprocal of the dissociation constant of the competitor (E_3). The inset is analogous exactly to the inset of Fig. 3-1: the points are plotted with the total concentration of bound sites (B_L+B_C) on the x-axis. In this "Scatchard format" the slope is $(-1/K_{dC})$ and the x-intercept is again B_0.


5.0
4.0
3.0
2.0
1.0
0.0
$\frac{B_C}{F_C}$
0.5
1.0
1.5
$[B_O - B_L - B_C]$ (nM)
10
8
6
4
2
0
$\frac{B_C}{F_C}$
0.5
1.0
1.5
2.0
2.5
$[B_L + B_C]$ (nM)

determined. For statistical reasons it may be advisable simply to apply the least squares criterion (i.e., to use the method of simple linear regression) and not force the line to pass through the origin. Since the plot of B_C/F_C vs. [free binding sites] is used to estimate only one binding parameter (K_{dC}), data sets arising from experimental preparations differing in total binding site concentration may be merged prior to the final analysis.

We have extended the analysis presented above to cover a very limited case of the two ligand-two binding site problem: the method has been used to "linearize" data resulting from investigation of the specific mouse brain glucocorticoid receptor in crude cytosol containing significant amounts of CBG (transcortin). We have measured the affinity of "cold" dexamethasone (which has negligible affinity for CBG) for the specific [^{3}H]corticosterone, non-CBG, binding sites (putative receptors) in CBG-containing brain cytosol in order to compare the resulting equilibrium constant with that obtained by using [^{3}H]dexamethasone itself to construct a one-ligand Scatchard plot. The experiment is performed by first constructing separate single-ligand binding isotherms using [^{3}H]dexamethasone and [^{3}H]corticosterone; then an isotherm describing the binding of [^{3}H]corticosterone in the presence of a fixed concentration of dexamethasone is constructed and analyzed. (The same cytosol preparation is used throughout, of course.) This analysis is simplified dramatically both by the absence of a significant interaction of dexamethasone with CBG and by the observation that the one-ligand [^{3}H]corticosterone Scatchard plot is not biphasic, which suggests that the affinity of corticosterone for CBG is very similar to its affinity for the putative receptors.

In order to describe the analysis we extend slightly the nomenclature used above; this nomenclature will apply only to the specific two-ligand, two-site isotherm to be "linearized." B_L and B_C are the total concentrations of specifically bound [^{3}H]corticosterone and "cold" dexamethasone, respectively. B_{LR} and B_{LT} are the concentrations of [^{3}H]corticosterone bound to the putative receptors and to CBG (transcortin), respectively. (Thus, $B_L = B_{LR} + B_{LT}$). B_{MAX} is the total concentration of all binding sites, composed of B_0 putative glucocorticoid receptors and T_0 transcortin binding site ($B_{MAX} = B_0 + T_0$). K_{dL} describes (approximately) the common affinity of [^{3}H]corticosterone for both the receptor and CBG binding sites, and K_{dC} is the equilibrium constant for the binding of dexamethasone to the glucocorticoid receptor sites. F_L and F_C are the concentrations of unbound (free) [^{3}H]corticosterone and "cold" dexamethasone, respectively.

From the mass-action equations (cf. equations 3-8 and 3-9 above) we have the following:

$$(B_0-B_{LR}-B_C)F_C = K_{dC}B_C, \tag{3-13}$$

$$(B_0-B_{LR}-B_C)F_L = K_{dL}B_{LR}, \tag{3-14}$$

$$(T_0-B_{LT})F_L = K_{dL}B_{LT} \tag{3-15}$$

and, adding equations (3-14) and (3-15) we get

$$(B_{MAX}-B_L-B_C)F_L = K_{dL}B_L. \tag{3-16}$$

Since dexamethasone does not bind to CBG, recall that the plot of B_C/F_C as ordinate vs. abscissa [$(B_0-B_{LR}-B_C)$, free receptor sites] is a line passing through the origin with slope $(1/K_{dC})$. This is the plot used to find K_{dC} for dexamethasone by competition with [^{3}H]corticosterone.

The calculation deriving K_{dC} from the competition data is straightforward. B_0 is determined from the one-ligand isotherm of [^{3}H]dexamethasone binding, and B_{MAX} and K_{dL} are derived from the one-ligand [^{3}H]corticosterone plot; T_0 is then estimated from the relation $T_0 = B_{MAX}-B_0$. The abscissa, [free glucocorticoid receptor sites], is now rewritten as

$$B_0-B_{LR}-B_C = (B_{MAX}-B_L-B_C) - (T_0-B_{LT}); \qquad (3\text{-}17)$$

i.e. [free receptor sites] = [total free sites]-[free CBG sites]. Substituting from equation (3-16) above we obtain for the abscissa

$$B_0-B_{LR}-B_C = (K_{dL}B_L/F_L) - (T_0-B_{LT}). \qquad (3\text{-}18)$$

Solving equation (3-15) for B_{LT} and substituting the resulting expression into equation (3-18) yields finally the computational form for the abscissa:

$$B_0-B_{LR}-B_C = K_{dL}[(B_L/F_L) - T_0/(F_L+K_{dL})]. \qquad (3\text{-}19)$$

Now only B_C must be found, since F_C and B_C/F_C follow immediately. The calculated value of the abscissa, [free receptor sites], is used to find B_C from the identity

$$B_C = B_0 - B_{LR} - [\text{free receptor sites}]. \qquad (3\text{-}20)$$

By substituting the expression for B_{LR} obtained from equation (3-14) into equation (3-20) above, the convenient computational form for B_C is found to be

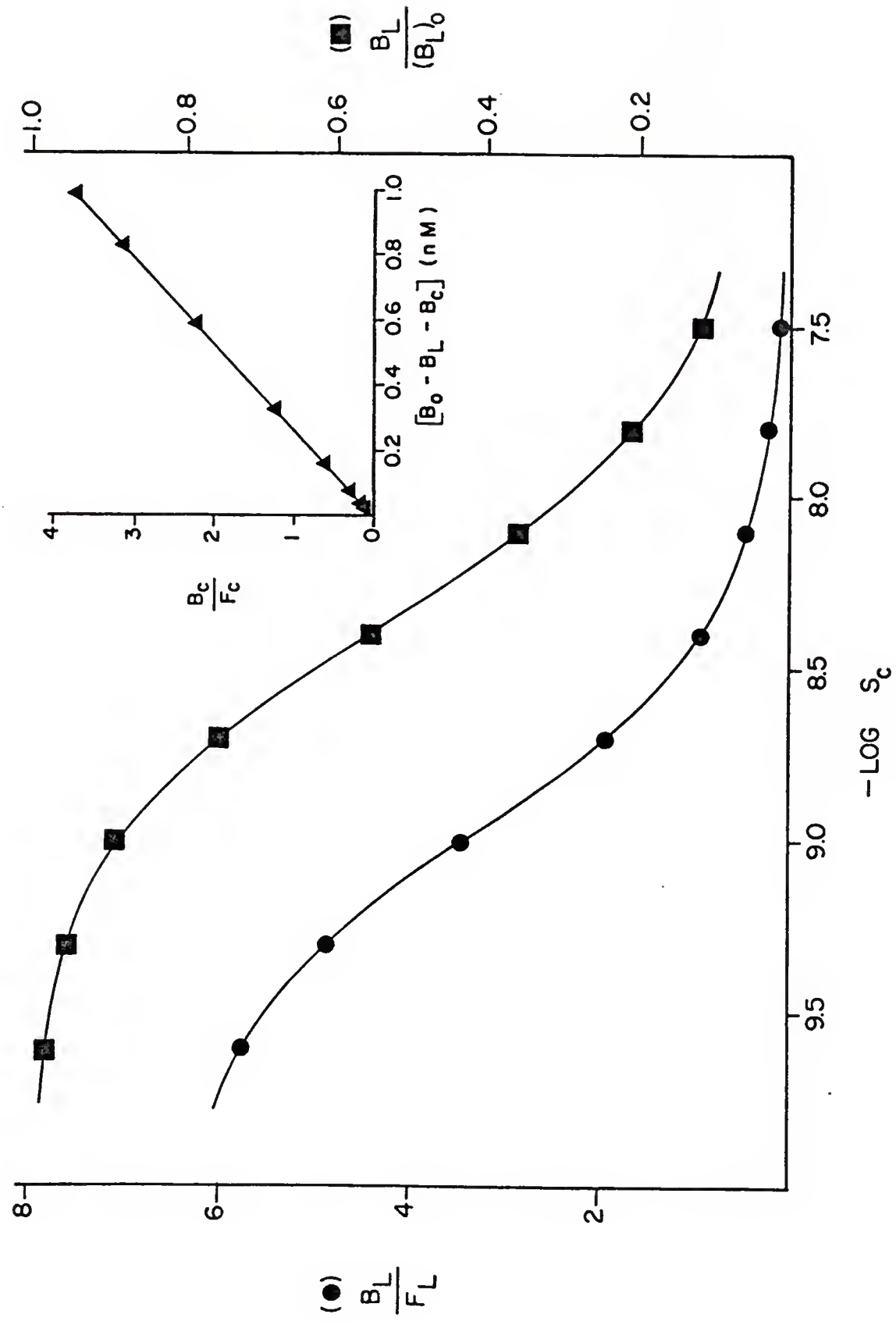
$$B_C = B_0 - [\text{free receptor sites}]\ (1+F_L/K_{dL}). \qquad (3\text{-}21)$$

The estimate of K_{dC} then follows directly from the slope of the recommended plot described above.

We now turn to an analysis of the "ED_{50}" experimental design. Figure 3-3 depicts theoretical competition displacement curves generated by the same binding parameters (taken from Weichman and Notides, 1980) that were employed in the construction of figs 3-1 and 3-2 and used in the above analysis of the design in which the inhibitor is present at a single concentration (the "Edsall-Wyman" design). Furthermore, it is presumed that K_{dL} and B_0 have previously been measured in the absence of competitor by constructing the one-ligand isotherm. If the initial receptor occupancy $[(B_L)_0/B_0]$ is not too high, it is obvious that linearization of the displacement curves may be achieved by plotting the data in the same B_L/F_C vs. $[B_0-B_L-B_C$, free binding sites] coordinate system discussed above in connection with the Edsall-Wyman experimental design, using the same data manipulations to determine the abscissa and ordinate of the plot (shown as inset to fig. 3-3). If the initial receptor occupancy is too high, then the range of the plot will be compressed severely and this method will not be useful.

Fig. 3-3. Theoretical curves for the competitive displacement of ligand L by ligand C from a single class of noninteracting binding sites.

Upper curve: fraction $B_L/(B_L)_0$ of initial bound ligand L remaining bound at equilibrium in the presence of different concentrations (S_C) of competitive inhibitor C; lower curve: the bound/free ratio for ligand L at the various concentrations of competing ligand C. Inset: linearization of the displacement curves by plotting the same data in the B_C/F_C vs [B_0-B_L-B_C, free binding sites] coordinate system. Again, the slope is $(1/K_{dC})$. The binding parameters used to generate the plots are listed in the legend to fig. 3-1 and are the same in all figures. The initial receptor occupancy depicted in the figure is 50%, and the concentrations of competitor C are spaced equally on a logarithmic scale.


(●) $\frac{B_L}{F_L}$
(■) $\frac{B_L}{(B_L)_o}$
$\frac{B_c}{F_c}$
$[B_o - B_L - B_c]$ (nM)
−LOG S_c

Other methods must be used to determine K_{dC} from the displacement plot when the initial receptor occupancy is too high. The direct estimation of an ED_{50} value from the curved displacement plot (fig. 3-3) is statistically naive, but a "pseudo-Hill" or logit-log transformation of the data (fig. 3-4) may be used to approximate a straight line for the estimation of ED_{50} by simple linear regression. The subsequent calculation of K_{dC} from the ED_{50} estimate presents further difficulties. We shall consider sequentially the problems of estimating ED_{50} and then using this estimate to calculate K_{dC}.

The "logistic" equations (see De Lean et al., 1978, for review; these are only approximations, as we shall demonstrate) that are used to transform the competition displacement data of fig. 3-3 into the approximately linear logit-log plots shown in fig. 3-4 are the following:

$$B_L = (B_L)_0/(1+S_C/ED_{50})^{1.0} \qquad (3\text{-}22)$$

and

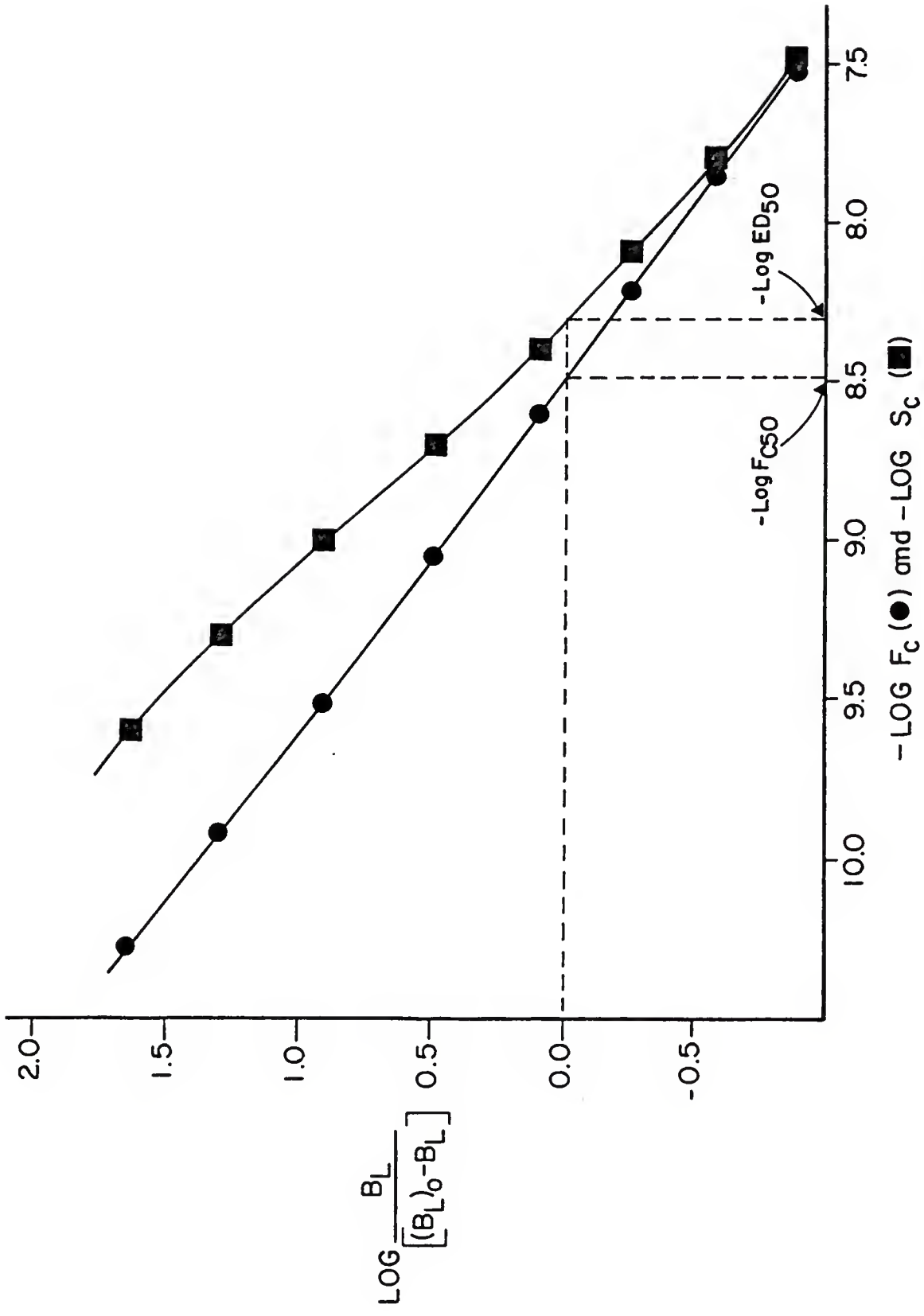
$$B_L = (B_L)_0/(1+F_C/F_{C50})^{1.0}. \qquad (3\text{-}23)$$

(The above Hill coefficient or "slope-factor" of 1.0 in the logistic equations is appropriate for non-interacting binding sites such as those under consideration here.) These logistic equations may be immediately transformed into the "pseudo-Hill" or logit-log expressions

$$\text{logit}\,[B_L/(B_L)_0] = \log\,(B_L/[(B_L)_0-B_L]) = \log ED_{50} - \log S_C \qquad (3\text{-}24)$$

Fig. 3-4. Logit-log transformations of the competitive displacement data shown in fig. 3-3.

Both curves share the same ordinate (logit $[B_L/(B_L)_0]$), but the abscissae refer respectively to the concentration of free competitor C (F_C, lower curve) and to the total concentration of ligand C (S_C, upper curve). The intersections with the dotted line (x-intercepts) define the ED_{50} and F_{C50} for the given conditions, but neither is a good estimate of K_{dC}. The slopes of the ED_{50} and F_{C50} plots determined by simple linear regression are, respectively, -1.22 and -0.91. The nonlinearity of the ED_{50} logit-log plot is quite apparent. The use of estimates of ED_{50} and F_{C50} to determine K_{dC} is discussed in the text.


2.0
1.5
1.0
0.5
0.0
-0.5
LOG $\frac{B_L}{[(B_L)_o - B_L]}$
10.0
9.5
9.0
8.5
8.0
7.5
-LOG F_c (●) and -LOG S_c (■)
-Log ED_{50}
-Log F_{C50}

and

$$\text{logit}\,[B_L/(B_L)_0] = \log\,(B_L/[(B_L)_0-B_L]) = \log F_{C50} - \log F_C, \tag{3-25}$$

which may be fit (approximately) to the binding data by simple linear regression. If equations (3-22) and (3-24) were exact, then the ED_{50} plot (upper curve, fig. 3-4) would be linear; if equations (3-23) and (3-25) were exact, then the F_{C50} plot (lower curve, fig. 3-4) would be linear. Equations (3-23) and (3-25) are (as we will show) always better approximations than equations (3-22) and (3-24). In the example under consideration equations (3-23) and (3-25) provide an excellent near-linear transformation of the binding data, as one can see upon examination of fig. 3-4; the nonlinearity of the ED_{50} plot, however, is quite apparent in this example.

We shall now derive the exact (but not computationally useful) expression for logit $[B_L/(B_L)_0]$ and then note the condition under which it may be approximated by equations (3-24) and (3-25). If the Edsall-Wyman equation (3-3, above) is combined with the obvious initial condition

$$(B_L)_0 = B_0(F_L)_0/[K_{dL}+(F_L)_0], \tag{3-26}$$

then we obtain, upon eliminating B_0 from equations (3-3) and (3-26),

$$B_L = C_1(B_L)_0F_L/[K_{dL}(1+F_C/K_{dC})+F_L], \tag{3-27}$$

where the constant $C_1 = [(F_L)_0 + K_{dL}]/(F_L)_0 = 1 + K_{dL}/(F_L)_0$. From equation (3-27) we immediately obtain

$$B_L/[(B_L)_0-B_L] = C_1F_L/[K_{dL}(1+F_C/K_{dC})+F_L(1-C_1)], \qquad (3\text{-}28)$$

which, upon elimination of C_1 from the denominator and division by F_LK_{dL}, yields

$$B_L/[(B_L)_0-B_L] = (C_1/K_{dL})/[(1/F_L)(1+F_C/K_{dC})-(1/(F_L)_0)]. \qquad (3\text{-}29)$$

Thus, the exact logit-log equation is given by

$$\text{logit}\,[B_L/(B_L)_0] = \log\,(C_1/K_{dL}) - \log\,[(1/F_L)(1+F_C/K_{dC})-(1/(F_L)_0)], \qquad (3\text{-}30)$$

which is not computationally useful (since the term for the logit-log abscissa itself contains the unknown K_{dC}). If, however, we may assume that F_L is approximately constant over the entire range of S_C (i.e., that $F_L \simeq (F_L)_0$), then equation (3-30) reduces to the simplified form

$$\text{logit}\,[B_L/(B_L)_0] \simeq \log\,[C_1K_{dC}(F_L)_0/K_{dL}] - \log\,F_C, \qquad (3\text{-}31)$$

which is the linear logit-log plot equivalent to equation (3-25) above. A comparison of equations (3-25) and (3-31) shows that the assumption $F_L \simeq (F_L)_0$ leads to the expression

$$F_{C50} \simeq C_1 K_{dC}(F_L)_0/K_{dL} = K_{dC}[1+(F_L)_0/K_{dL}], \qquad (3\text{-}32)$$

thus indicating clearly that F_{C50} (and, of course, ED_{50}) is quite different from the desired parameter K_{dC}, which must be determined by an additional calculation. Although the above approximation that leads to linearization of the logit-log F_C plot (i.e., $F_L \simeq (F_L)_0$) is derived from the initial occupancy condition $(B_L)_0 << S_L$, the approximate linearity of the plot is fairly robust over a broad spectrum of experimental conditions and depends only on the initial conditions relating to the labeled ligand L. Specifically, the approximate linearity of the logit-log F_C plot does not depend on the relative affinity of the two ligands, (K_{dC}/K_{dL}). The logit-log plot containing log S_C as abscissa (the "ED_{50}" logit-log S_C plot), however, departs significantly from linearity because the approximation $F_C \simeq S_C$ is a poor one at low values of S_C. If $K_{dC} >> K_{dL}$ then the large values of S_C required to achieve ligand displacement will also make this formula approximately valid and thus lead to linearization of the simpler ED_{50} plot. The calculation of F_C for the construction of the logit-log F_C plot from the measured data has been described above (equation 3-12 combined with the relation $F_C = S_C - B_C$), and the initial binding $(B_L)_0$ may either be measured directly or calculated from the values of K_{dL} and B_0 (in combination with the known S_L) measured previously. In the specific example under consideration simple linear regressions of the theoretical logit-log data of fig. 3-4 yield the following results: [ED_{50} (lin. regress.)/"true" ED_{50}] = 1.12 (13% error), and [F_{C50} (lin. regress.)/"true" F_{C50}] = 0.999 (0.1% error). (The "true" values of ED_{50} and F_{C50} are, respectively, 4.80 and 3.20 nM.)

Further analysis is, of course, required to calculate K_{dC} from the estimates of either ED_{50} or F_{C50} obtained from the logit-log plots discussed above. If, in equation (3-32) above, F_{C50} is replaced by ED_{50} and $(F_L)_0$ by S_L, one obtains the Cheng-Prusoff (Cheng and Prusoff, 1973; Munson and Rodbard, 1980) correction

$$K_{dC} \simeq ED_{50}/(1+S_L/K_{dL}). \tag{3-33}$$

This formula is derived from equations (3-2) and (3-3) above by using the definition of ED_{50} (i.e., that $(B_L)_0 = 2B_L$ when $S_C = ED_{50}$) and applying the drastic approximation that both $F_C \simeq S_C$ and $F_L \simeq S_L$. As the illustrative example will demonstrate, this does not provide a good estimate of K_{dC} when the affinity of the competing ligand is too high.

We now show that this Cheng-Prusoff correction can be improved substantially by including in the calculation the value of $(B_L)_0$, which easily can be measured experimentally or calculated from the values of K_{dL} and B_0 measured previously. Combining the above equations (3-3), (3-23) and (3-26) yields, upon elimination of B_0, the expression

$$(F_L)_0F_{C50}/[K_{dL} + (F_L)_0][F_{C50}+F_C] \simeq F_L/[K_{dL}(1+F_C/K_{dC})+F_L], \tag{3-34}$$

which, when evaluated at the 50% displacement point, becomes

$$(F_L)_0/2[K_{dL}+(F_L)_0] \simeq F_{L50}/[K_{dL}(1+F_{C50}/K_{dC})+F_{L50}]. \tag{3-35}$$

(Expressions (3-34) and (3-35) above are not exact, since they are derived from the logistic equation (3-23), which is itself only an approximation.) Since $F_{L50} = S_L - (B_L)_0/2$ and $(F_L)_0 = S_L - (B_L)_0$ we finally obtain, after elimination of F_{L50} and $(F_L)_0$ and some rearrangement and simplification,

$$K_{dC} \simeq F_{C50} \times 2K_{dL}[S_L-(B_L)_0]/[(B_L)_0^2 + 2S_L^2 + 2S_L K_{dL} - 3S_L(B_L)_0]. \qquad (3\text{-}36)$$

This is a much better approximation than the Cheng-Prusoff equation (3-33), to which it reduces when $(B_L)_0$ is neglected and F_{C50} is replaced by ED_{50}. Equation (3-36) remains approximately valid, and is still superior to equation (3-33), when ED_{50} is substituted for F_{C50}:

$$K_{dC} \simeq ED_{50} \times 2K_{dL}[S_L-(B_L)_0]/[(B_L)_0^2 + 2S_L^2 + 2S_L K_{dL} - 3S_L(B_L)_0]. \qquad (3\text{-}37)$$

Equation (3-36) will, however, always be superior to equation (3-37); similarly, the Cheng-Prusoff expression itself will always be more nearly exact if a good estimate of F_{C50} is substituted for the estimate of ED_{50}. Table 3-1 lists, for the example that we have been considering, the K_{dC} estimates derived from the two different approximations; each method has been used in combination with both the estimated and the exact values of F_{C50} and ED_{50} listed above. It will be seen that the retention of $(B_L)_0$ in the approximation is required for

Table 31. Estimates of K_{dC} (The assumed exact value is 2.632×10^{-10}M) derived from the Cheng-Prusoff correction and from the approximations described by equations 3-36 and 3-37. The inputs to the equations include both the exact values of F_{C50} and ED_{50} for the example discussed in the text and the estimated values derived as described from the logit-log plots of Fig. 3-4.

	Input ED_{50}		Input F_{C50}	
	Exact	Estimated	Exact	Estimated
Cheng-Prusoff method (eqn. 3-33)	5.462×10^{-10}M	6.107×10^{-10}M	3.648×10^{-10}M	3.644×10^{-10}M
Method of eqns. (3-36) & (3-37)	3.941×10^{-10}M	4.406×10^{-10}M	2.632×10^{-10}M	2.629×10^{-10}M

the accurate derivation of K_{dC} from the competition displacement data under consideration here. The ease of implementation of equation (3-36) suggests that it (or at least equation 3-37) should always be used instead of the Cheng-Prusoff approximation.

Although the graphical linearization of competition data is achieved most conveniently in the recommended coordinate systems discussed above, it can be displayed in simple modifications of any of the popular binding plots. For example, a modified "Lineweaver-Burke" plot (Lineweaver and Burke, 1934) that is linear with slope K_{dC} (and y-intercept 1.0) can be constructed by plotting $(B_0-B_L)/B_C$ on the ordinate with $1/F_C$ on the abscissa. A modified "direct linear" plot (Eisenthal and Cornish-Bowden, 1974) may even be used to estimate K_{dC} by finding the median of the abscissae of the intersections where lines plotted for each of the individual observations in the usual "direct linear" parameter space (F_C, B_C) intersect the horizontal lines having ordinates B_0-B_L.

The problem of determining the best-fitting line for the recommended B_C/F_C vs. $[B_0-B_L-B_C$, (free binding sites)] plot is similar to the problem of regression for the original one-ligand Scatchard plot and has, in this context, been adequately discussed (e.g. Cressie and Keightley, 1979; Rodbard, 1973; Rodbard and Feldman, 1975). In addition, by the very nature of the definition of logit $[B_L/(B_L)_0]$, the logit-log plots of competition displacement data are quite sensitive to error in the measurements of B_L performed at the low concentrations of the competing ligand. Although the assumptions underlying the use of the method of least squares (e.g., uniformity of variance, noncorrelation of error in the independent and dependent variables) are

clearly violated in both the Scatchard and logit-log transformations of the data (e.g., Rodbard, 1973), the method of least square is still used frequently as a convenient first approximation and is probably not too seriously biased if "outliers" are few and if confidence intervals are strengthened by repetition of the experiment. Thus, the method of simple linear regression or of linear regression through a fixed point (the origin) may be applied to the recommended B_C/F_C vs [free binding sites] plot; the calculation for the latter (Pollard, 1977) is routine and directly yields

$$K_{dC} = 1/\text{slope} = K_{dL}\Sigma_i(B_L/F_L)_i/\Sigma_i(B_L/F_L)_i(B_C/F_C)_i. \qquad (3\text{-}38)$$

The approximate analysis of variance and confidence intervals are given in standard tests (e.g., Pollard, 1977). It is also convenient to use the simple and more "robust" median parameter estimates discussed by Cressie and Keightley (1979). This procedure (Cressie and Keightley, 1979) may be used to determine K_{dL} and B_0 from the initial Scatchard plot; and then if the line is to be forced to pass through the origin, the "free receptor" plot for the direct determination of K_{dC} may be analyzed by calculating the median estimate

$$K_{dC} = 1/\text{slope} = \text{median of } (B_L K_{dL} F_C/B_C F_L)_i. \qquad (3\text{-}39)$$

In any case, the statistical complexity of the experiment demands that confidence in the parameter estimates obtained by any of the methods discussed above must come from replication of the complete design.

Discussion

A simple procedure for linearizing the curved Scatchard plot of the binding of a labeled ligand to a single class of noninteracting binding sites in the presence of a fixed total concentration of competitive inhibitor has been presented. Since the nonlinearity of the Scatchard plot constructed in the presence of the inhibitor may not be apparent upon visual inspection because of variance in the data, the method of calculating the equilibrium constant of the competitive inhibitor recommended above should be used unless it is known that the equilibrium dissociation constant of the inhibitor (K_{dC}) is much larger than the total concentration of binding sites. The same procedure may also be applied to the analysis of data derived from the "ED_{50}" competition displacement experimental design (if the initial receptor occupancy is not too high). In addition, a useful approximation (a generalization of the Cheng-Prusoff formula) that may be used to relate the ED_{50} or F_{C50} estimates obtained from approximately linear logit-log plots of competition displacement data to the actual affinity of the competitive inhibitor for the binding sites has been presented.

Significant sources of error remain inherent in both experimental designs and cannot be eliminated easily; these include nonspecific binding of the competitive inhibitor and also the potential presence of additional receptor sites having significant affinity for the competitive inhibitor but negligible affinity for the labeled ligand. Furthermore, the statistical characteristics of the simple graphical methods suggested above currently remain untested and must eventually be examined in detail by the Monte-Carlo simulation procedure (e.g. Thakur,

Jaffe and Rodbard, 1980); for example, the competition "free receptor" plot is probably less sensitive to error in B_0 and K_{dL} when it is "relaxed" (i.e., not forced to pass through the origin). Whenever practical, a computer program (e.g., Munson and Rodbard, 1980) should be used to fit binding isotherms by a weighted, nonlinear regression technique performed with a relatively error-free independent variable. The plot of B_C/F_C vs [free binding sites] or the competitive displacement logit-log plot may still be used to display the actual data points and the fit of the resulting computer-generated parameters.

CHAPTER IV

EQUILIBRIUM BINDING CHARACTERISTICS AND HYDRODYNAMIC PARAMETERS OF MOUSE BRAIN GLUCOCORTICOID BINDING SITES

Introduction

Glucocorticoids have profound metabolic, neuroendocrine, and behavioral effects in the mammalian brain (for reviews, see: Bohus et al., 1982; Rees and Gray, 1982). Although some of the less-well-understood effects may result from direct interactions of the steroid with components of target cell membranes, many of the effects are thought to be mediated by interactions of the hormone molecules with steroid-specific cytoplasmic and nuclear macromolecular receptors that concentrate as activated hormone-receptor complexes in the target cell nuclei, where they initiate changes in gene expression that produce the ultimate physiological effects.

The experiments reported here examine a number of physiochemical characteristics of soluble mouse brain glucocorticoid binding sites, in order to determine whether the glucocorticoid receptor system in mouse brain resembles closely that operative in other target tissues and species. Although a few published studies have reported the existence of glucocorticoid receptors in the mouse brain (e.g., Finch and Latham, 1974; Nelson et al., 1976; Angelucci et al., 1980), no basic characterization of the kinetic and equilibrium binding parameters or the steroid specificity of these receptors has yet been reported.

Although a body of literature concerned with the properties of rat brain glucocorticoid receptors already exists (for review, see: Bohus et al., 1982), recent improvements in receptor methodology have made it possible to study the brain glucocorticoid receptor system under conditions that prevent receptor activation (nucleophilic transformation) and maximize in vitro receptor stability, permitting the relatively lengthy incubations and procedures required to generate equilibrium isotherms and to investigate the size and shape of the receptors.

We have used the labeled glucocorticoids [^{3}H]corticosterone, [^{3}H]dexamethasone and [^{3}H]triamcinolone acetonide (cyclic acetal). [^{3}H]Dexamethasone and [^{3}H]corticosterone were used to measure equilibrium and kinetic binding parameters, whereas the nearly-irreversible ligand [^{3}H]triamcinolone acetonide ([^{3}H]TA) was used for the lengthy sedimentation and chromatography procedures required to examine receptor size and shape. The experiments reported here have explored buffer components and employed a buffer that prevents the loss of unoccupied binding sites at 2°C; used a rapid and convenient binding assay; considered the possible consequences of failure to allow adequate incubation time when ligand concentrations are low; compared equilibrium binding parameters derived from the same pool of experimental data by several different methods of analysis; determined ligand specificity by applying mathematically correct procedures to the analysis of steroid competition data; observed that CBG-like molecules contribute signficantly to the total pool of corticosterone binding sites, and examined binding site sizes and shapes to assess the stability and homogeneity of the in vitro receptor population.

Materials and Methods

Chemicals, Steroids and Isotopes

The [1,2,6,7-^{3}H]corticosterone (SA = 80 Ci/mmole), [6,7-^{3}H]dexamethasone (SA = 36 Ci/mmole) and [6,7-^{3}H]triamcinolone acetonide (SA = 37 Ci/mmole) were purchased from New England Nuclear (Boston, MA) and checked for purity by chromatography on 60 cm LH-20 columns (Sippell, Lehmann and Hollmann, 1975) or by thin layer chromatography (TLC) on Silica Gel G (plates were developed in cyclohexane : methyl ethyl ketone, 1:1, or in dichloromethane : methanol, 24:1). All nonradioactive steroids were purchased from Steraloids, Inc. (Wilton, NH). Additional radiochemicals ([^{14}C]antipyrene, [2-^{3}H]deoxy-D-glucose, [^{14}C]formaldehyde, [carboxyl-^{14}C]inulin and [^{3}H]water) and a [^{125}I]cortisol Solid Phase Radioimmunoassay kit were purchased from New England Nuclear. Chromatography and filtration supplies were purchased from Pharmacia Fine Chemicals (Piscataway, NJ), Bio-Rad Laboratories (Richmond, CA), Whatman, Inc. (Clifton, NJ), Brinkmann Instruments, Inc. (Westbury, NY), and the Amicon Corp. (Danvers, MA). Rabbit antiserum to corticosterone was a gift from R.H. Underwood and G.H. Williams (Peter Bent Brigham Hospital, Boston, MA). Other chemicals and solvents were of the highest purity available commercially.

Animals

All studies used adult female CD-1 mice (outbred, 20-25g Charles River Laboratories, Wilmington, MA) that were subjected to combined ovariectomy and adrenalectomy 3-5 days prior to the experiment in order

to remove known sources of endogenous steriods. Ovariectomy and adrenalectomy were performed bilaterally via a lateral, subcostal approach under Nembutal anesthesia, and mice were given 0.9% NaCl in place of drinking water. On the day of the experiment mice were anesthetized with ether (in some cases a .5-1 ml blood sample was then withdrawn directly from the heart) and slowly perfused (over a period of 5 min) through the heart with cold HEPES-buffered saline (3 ml, isotonic, pH 7.6) to reduce blood-borne CBG contamination of the brain tissue. The efficacy of this procedure was assessed by a [^{14}C]inulin washout study (see Chapter V).

The effectiveness of the surgery was verified by measurement of corticosterone levels in plasma samples obtained from some of the adrenalectomized mice at the time of killing. This was accomplished by radioimmunoassay (RIA) both with rabbit antiserum to corticosterone (by a modification of the method of Underwood and Williams, 1972) and with the New England Nuclear [^{125}I]Cortisol solid phase RIA kit using corticosterone to construct the standard curve. Plasma samples (10μl) were extracted with 1 ml dichloromethane and then dried down prior to RIA.

Buffers

For most of the experiments cytosol was prepared in buffer A: 20 mM HEPES, 1 mM EDTA, 2 mM DTT, 10 mM Na_2MoO_4, 10% (w:v) glycerol, pH 7.6. The effects of pH and of the dithiothreitol (DTT) and molybdate (Na_2MoO_4) concentrations on binding site stability were explored in several initial experiments, and gel filtration and sedimentation were performed at elevated ionic strength (with the addition of KCl). In the descriptions of these experiments buffer compositions are reported as

modifications of the basic buffer A formulation (e.g., buffer A - DTT, buffer A + .15M KCl, etc.).

Cytosol Preparation and Aging

Brains were removed from the perfused animals and homogenized (20 strokes at 1000 rev/min) in 1-6 volumes of cold buffer A in a glass homogenizer with a Teflon pestle milled to a clearance between the pestle and homogenization tube of 0.125 mm on the radius (to minimize rupture of the brain cell nuclei, as suggested by McEwen and Zigmond, 1972). The crude homogenate was centrifuged at 2°C for 10 min at 15,000 rpm (27,000 x g) in a 15 ml Corex centrifuge tube. The supernatant was transferred to a 10 ml "Oak Ridge" polycarbonate tube, and the pellet was washed by resuspension in half the initial volume of buffer A (used for the homogenization) and recentrifugation at 2 C for 10 min x 27,000 g. The resulting supernatant wash was added to the original supernatant in the "Oak Ridge" tube, which was then centrifuged at 2 C for 1 h at 106,000 x g (average) to produce the cytosol used in the incubations. Approximately 2 h elapsed between the time of killing and termination of the high-speed centrifugation. Cytosol protein concentrations for the equilibrium experiments ranged from approximately 2 mg/ml (with homogenization in 6 volumes buffer A, initial pellet then washed with 3 volumes buffer A) to 12 mg/ml (homogenization in 1 volume buffer A, pellet washed with 0.5 volume additional buffer A). The cytosol protein content was determined by a modification of the Lowry method (Bailey, 1967) using bovine serum albumin as the standard.

In several experiments cytosol samples were incubated without steroid for variable lengths of time before labeling with the

[^{3}H]steroid ligand (i.e., the cytosol was deliberately "aged" beyond the approximately 2 h required for cytosol preparation). In such experiments the duration of aging was measured from the time when cytosol preparation was completed (t = 0).

In order to examine the subcellular distribution of glucocorticoid binding sites in mouse brain the standard subcellular fractionation scheme (Cotman, 1974) was employed to generated crude nuclear (P_1), crude mitochondrial (P_2), and microsomal (P_3) fractions in addition to the cytosol; the fractionation scheme was modified, in that buffer A (which is hypertonic because it contains 10% glycerol) was used instead of isotonic .32 M sucrose. The concentrations of high-affinity glucocorticoid binding sites measured in the subcellular fractions (other than cytosol) generated in this way were negligible, and thus these hypertonically-produced particulate fractions were not further characterized.

Binding Assays

The principal assay used to measure bound [^{3}H]steroid was the DEAE (Whatman DE-81) filter assay, modified from similar assays developed to measure glucocorticoid and mineralocorticoid binding to cytosolic proteins in liver and kidney (e.g., Warnock and Edelman, 1978; Santi, Sibley, Perriard, Tomkins and Baxter, 1973). The 25 mm filters were equilibrated at 0-4°C in buffer A and then washed 2 times with 2 ml buffer A in a Millipore suction manifold. For each of the triplicate assays that commonly were performed a 50 µl aliquot of the sample was pipeted directly onto the moist filter and allowed to penetrate it for at least 1 min. Filters were then washed with 5 x 1 ml buffer A,

suctioned to near dryness, and then transferred to scintillation vials. Filtration was performed at 0-4°C in a cold room. Radioactivity was determined by liquid scintillation counting at 38% efficiency following the addition of 1 ml H_2O and 10 ml Triton-toluene scintillation cocktail (toluene-Triton X-100, 2:1; 2, 5-diphenyloxazole, 4.375 g/l; dimethyl POPOP, 43.75 mg/l) and disruption of the filters by vortexing of the vials. Alternatively, the filters were dried overnight in a warm oven and then counted at higher efficiency in a toluene based scintillation cocktail not containing Triton X-100. The efficiency of the DEAE filter assay was determined (as described below) by two different methods and found to be 76%.

In order to calculate the efficiency of the DEAE filter assay some of the binding data obtained with it were compared with those obtained from the same samples with a Sephadex G-25 minicolumn gel filtration assay and with a dextran-charcoal adsorption assay. In the former procedure 50 µl aliquots of the sample were loaded onto Pasteur pipet minicolumns filled with 1.5 ml Sephadex G-25 equilibrated in buffer A; following collection of a 0.51 ml fraction that was discarded, a 0.8 ml void volume containing bound radioactivity was collected and assayed by liquid scintillation in a Triton-toluene cocktail. The dextran-charcoal adsorption assay was performed by adding the 50 µl sample aliquot to a 1 ml suspension of dextran-coated charcoal (0.5% Norit A activated charcoal, Fisher; 0.05% Dextran T-70, Pharmacia); following incubation at 0-4°C for 5 min with occasional vortexing the charcoal was pelleted by centrifugation at 10,000 x g for 5 min. The supernatant was then taken for the determination of bound radioactivity by liquid scintillation counting.

Gel Chromatography and Gradient Sedimentation

The in vitro stability and molecular size of the receptors were examined by Sephacryl S-300 chromatography and glycerol gradient sedimentation. Concentrated cytosol prepared in buffer A + 150 mM KCl was incubated at 2°C for 12-16 h with 10 nM [^{3}H]TA or 10 nM [^{3}H]TA and 200-fold excess of unlabeled TA. Following incubation, free [^{3}H]TA was removed by gel filtration in the same buffer on a large (1.5 x 98 cm) Sephadex G-25 (fine) column. Samples from the void volume of the G-25 column (2 ml, approximately 5 mg/ml) were then chromatographed on a column of Sephacryl S-300 (superfine, 1.5 x 98 cm) at 12 ml/h in the same buffer (A + .15 M KCl). Fractions (2 ml) were collected, and 1 ml aliquots of these were assayed for radioactivity by liquid scintillation. (The remaining 1 ml from the relevant fractions was saved for gradient sedimentation.) Distribution coefficients (K_d) were calculated from elution volumes (V_e), the void volume (V_o) indicated by blue dextran, and the total liquid volume (V_i) marked by [^{3}H]water, [^{14}C]antipyrene and [2-^{3}H]deoxy(D)glucose: $K_d = (V_e - V_o)/(V_i - V_o)$. The column was calibrated (figure 4-29) with 7 standard proteins (chromatographed in separate runs as two different mixtures and detected by absorbance at 280 nm), and the Stokes radius of the receptor complex (R_s) was calculated from regressions of R_s vs. several different functions of the calibration K_d values as shown in figure 4-30. Standard curves were generated by the linear methods of Porath (1963) and Ackers (1967), and by the nonlinear regression of Laurent and Killander (1964). The methods of regression were in close agreement.

After locating the peak of the gel chromatographic profile, the remaining 1 ml aliquots from each of the 5 fractions bracketing the

S-300 peak were pooled and concentrated 10-fold by ultrafiltration (Amicon Minicon B-15 or Model 12 Ultrafiltration cell with PM 10 membrane) to a volume of 500 µl. Aliquots (250 µl) of the concentrated, pooled S-300 fractions were then centrifuged at 2°C for 20 h at 234,000 g (average) through 5 ml gradients of 12.5 - 25% (w/v) glycerol or 15 - 50% (w/v) glycerol prepared in the same buffer (A + .15 M KCl). The cellulose nitrate tubes were punctured, and 175 µl fractions were collected and assayed for radioactivity. The value of $S_{20,w}$ for a peak was calculated from the linear regression of $S_{20,w}$ vs. sedimentation distance (Martin and Ames, 1961) for the standard proteins, which were run on parallel gradients as 250 µl samples in the same buffer (A + .15 M KCl). The standard proteins ovalbumin (3.6 S), bovine serum albumin (BSA, 4.3 S) and IgG (γ-globulin, 7.4 S) were [$^{14}CH_3$]-methylated (for detection) to low specific activity by the method of Rice and Means (1971).

Assuming that peaks observed on the glycerol gradients corresponded to the same molecular species resolved by S-300 chromatography, the apparent sizes and shapes of the binding sites were determined by standard methods (Siegel and Monty, 1966; Sherman, 1975). The Stokes radius (R_s) and sedimentation coefficient ($S_{20,w}$) of the protein are related to its apparent molecular weight by the relation

$$M = 6\pi N \eta_{20} \times R_s \times S_{20,w}/(1 - \bar{\nu}\rho_{20,w}), \qquad (4\text{-}1)$$

where the assumed value of the partial specific volume ($\bar{\nu}$ = 0.732 cm^3/g) was estimated as described previously by Sherman (1975). The viscosity of H_2O at 20°C (η_{20}) = 0.01002 poise (g. $cm^{-1}.sec^{-1}$), and the density of

H_2O at 20°C ($\rho_{20,w}$) = 0.9982 g/cm^3; and Avogadro's number (N) = 6.023 x 10^{23} /mole. The frictional ratio was determined from the relation

$$f/f_o = R_s[4\pi N/3M(\bar{\nu} + \delta\bar{\nu}_{20,w})]^{1/3}, \quad (4\text{-}2)$$

where the partial specific volume of H_2O at 20°C ($\bar{\nu}_{20,w}$) = 1.00 cm^3/g and the hydration state of the protein (δ) was taken to be either 0 (i.e., no hydration) or δ = 0.2 g/g, an estimate used to permit comparison of our results with those reported by Niu, Neal, Pierce and Sherman (1981). The axial ratios (b/a, prolate ellipsoid) were calculated from the Perrin equation

$$f/f_o = (1 - p^2)^{\frac{1}{2}}/p^{2/3}\ln([1 + (1 - p^2)^{\frac{1}{2}}]/p), \quad (4\text{-}3)$$

where p = b/a = ratio of minor to major axes of the prolate ellipsoid; some solutions to Perrin's equation have been tabulated by Schachman (1959).

DEAE and Dye-Ligand Minicolumn Chromatography

Cytosol samples labeled with [^{3}H]TA were chromatographed on Bio-Rad DEAE Bio-Gel A minicolumns (column characteristics: total volume = 5 ml; flow rate = 6 ml/h; fraction volume = 1 ml; maintained at 4°C). The column was washed extensively and equilibrated with buffer A. Concentrated cytosol prepared in buffer A was incubated at 2°C for 12-16 h with 10 nM [^{3}H]TA or 10 nM [^{3}H]TA and 200-fold excess unlabeled TA. Following incubation, free [^{3}H]TA was removed by gel filtration in buffer A on a large (1.5 x 98 cm) Sephadex G-25 (fine) column. Samples

from the void volume of this column (1 ml, approximately 5 mg/ml) were run into the DEAE minicolumn and washed in with 200 µl buffer A. Binding was allowed to proceed (30 min), and the column was then washed with 25 ml buffer A. Bound species were eluted with a 50 ml linear KCl gradient (0 - 250 mM KCl in buffer A); during column wash and elution fractions were collected and assayed for radioactivity.

The potential binding of [^{3}H]TA-receptor complexes to immobilized triazine dyes was assessed with an Amicon Dyematrex screening kit consisting of five different dye-agarose minimcolumns (and an agarose control column). The columns (2 ml volume) were washed extensively and equilibrated with buffer A. Following the removal of unbound [^{3}H]TA, cytosol samples (.5 ml, protein concentration 5mg/ml) equilibrated previously with 10 nM [^{3}H]TA were applied to the columns and washed in with .1 ml buffer A. Binding was allowed to proceed (30 min), and the columns were then eluted (stepwise) with the following series of reagents (10 ml each, in buffer A): buffer A alone, 5 x 10^{-5} M dexamethasone, 5 x 10^{-5} M progesterone, 10 mM ATP, 10 mM NADP, 130 mM KCl, 380 mM KCl, 2M urea + .25M NaSCN, 8M urea + .5M NaOH (no buffer). The nine 10 ml fractions were collected and assayed for radioactivity.

Experimental Designs and Data Analyses

Equilibrium isotherms were generated by incubating a series of pairs of identical cytosol samples (for a time sufficient to allow a close approach to equilibrium in all tubes) at 2°C with increasing concentrations of [^{3}H] glucocorticoid and, for one of each sample pair, a 200-fold excess of the unlabeled steroid. The problem of choosing an incubation time that is adequate to allow close approach to equilibrium

but is not excessive has been discussed in some detail in chapter II and will be addressed again below in Results. Bound [^{3}H]steroid concentrations were then assayed in triplicate for both tubes of each pair in order to determine total binding (B_T) and nonspecific binding (B_{NS}, binding in the presence of the 200-fold excess unlabeled steroid). Specifically bound steroid (B_{sp}) was determined for each [^{3}H]ligand concentration by subtracting B_{NS} from B_T. The values of B_T and B_{sp} were corrected for the measured efficiency of the DEAE filter assay (76%, see Results below) before the data were further analyzed. Since for reasons of signal strength it was not desirable to restrict the concentration of binding sites such that bound ligand did not exceed a small fraction of the total added ligand (S_L), further analysis of the data by conventional methods required the calculation of the free [^{3}H]ligand concentration (F), which was found by subtracting B_T from the total [^{3}H]ligand (S_L), which was also measured for each pair of tubes.

Equilibrium isotherms were generated with the synthetic agonists [^{3}H]dexamethasone and [^{3}H]TA in order to measure the concentration of putative glucocorticoid receptors and their affinity (or affinities) for these ligands. Isotherms were constructed with [^{3}H]corticosterone (and excess unlabeled corticosterone) to examine at once the complete ensemble of binding sites for the natural glucocorticoid, a subset of which resembled CBG and failed to bind DEX and TA. The binding of [^{3}H]corticosterone itself to the restricted set of putative glucocorticoid receptors was examined by constructing isotherms in which the contribution of dexamethasone-resistant (CBG-like) binding sites was removed from the determination of specific binding (B_{sp}) by using 200X unlabeled DEX to saturate only the putative glucocorticoid receptors in

the "nonspecific" control tubes, allowing any corticosterone-specific or otherwise CBG-like binders to remain labeled with [^{3}H]corticosterone. In several experiments (e.g., the study of the loss of unocupied binding sites as a function of cytosol aging and temperature) the concentration of binding sites was measured not by extrapolation from isotherms but rather by single-point assays performed following incubation with concentrations of [^{3}H]ligand known to be nearly saturating; these assays were also performed in triplicate with pairs of tubes containing [^{3}H]ligand and [^{3}H]ligand + 200 X unlabeled steroid.

Equilibrium isotherms were analyzed by several different methods. First, the values of B_{sp} and F were used to construct conventional Scatchard plots to which, when the points appeared to fall approximately on a straight line (and thus to define a single class of noninteracting binding sites), lines were fit by simple 2-parameter linear regression according to the equation

$$B_{sp}/F = -(B_{sp} - B_0)/K_d, \qquad (4\text{-}4)$$

where K_d is the equilibrium dissocation constant and B_0 is the molar concentration of binding sites; the slope and x-intercept of this plot thus provide estimates of the affinity and binding site concentration when a single ligand binds to a single class of noninteracting binding sites.

It would be desirable to combine a number of separate isotherm data sets constructed with cytosol pools having different protein concentrations in a "merged Scatchard" analysis. In such an analysis each value of B_{sp} (expressed originally in molar units) would be

normalized by the appropriate protein concentration and thus converted to the units fmole/mg protein; the free ligand concentration (F) would remain in molar units (e.g., nM). Following normalization of the B_{sp} values the Scatchard plot would be constructed by plotting all points in the conventional fashion, with the ordinate (B_{sp}/F) now possessing the units (fmole/mg protein)/nM and the abscissa (B_{sp}) having the units fmole/mg protein. The regression line would then be fit by simple 2-parameter linear regression. This "merged Scatchard" plot could be used to cumulatively analyze data from an experiment that has been repeated many times; the underlying assumption is, of course, that the functional receptor concentration and ligand affinity do not depend upon the cytosol concentration.

Because the estimates of functional receptor concentration (B_0) derived from individual Scatchard plots for different pools of cytosol were highly variable, the method described above was not used. Instead, a simple modification of the "merged Scatchard" plot that obviated the above assumption was used to obtain a single, merged estimate of K_d for comparison with estimates produced by other methods. The ordinate of the resulting "free receptor" plot (e.g., figure 4-20) was the same as that of the conventional Scatchard plot, but the abscissa was changed from bound (B_{sp}) to free receptor concentration (B_0-B_{sp}) by subtracting each value of B_{sp} from the corresponding value of B_0 derived by Scatchard analysis of the complete isotherm containing the input data point. A line (which theoretically should satisfy the Scatchard equation 4-4 and pass through the origin) was fit by 2-parameter least-squares linear regression and thus was not constrained to intersect the origin. The line was actually fit by a computerized

non-linear regression program (Helwig and Council, 1979) that generated a standard error of the mean ("asymptotic" s.e.m.; Ralston and Jennrich, 1978) for the merged estimate of the parameter K_d, the reciprocal of the slope of the "free receptor" plot. This unorthodox s.e.m. was never used to derive an inferential statistic; however, it did provide an estimate of the variability within the total pool of data.

The estimate of B_0 derived from the Scatchard plot for each individual isotherm was also used, in conjunction with the individual values of B_{sp}, to construct the Hill plot for the particular experiment according to the Hill equation

$$\text{logit } \theta = \log[\theta/(1-\theta)] = n \log (F/K_d), \qquad (4\text{-}5)$$

where θ is the binding site occupancy (B_{sp}/B_0) and n, the exponent of F in the basic isotherm equation $B_{sp} = B_0F^n/(K_d + F^n)$, is the Hill coefficient, which is 1.0 for a single class of noninteracting binding sites. A line was fit by linear regression to the points whose occupancy (θ) did not exceed 80% (i.e., logit $\theta \leq 0.6$), and the slope of this line was taken as the Hill coefficient. A slope significantly different from 1 in the neighborhood of the x-axis of a Hill plot (where logit θ = 0) is indicative of apparent binding cooperativity or some other, artifactual source of curvature in the initial Scatchard plot. Although seldom used as such, the value of F at the x-intercept is an estimate of the dissociation constant K_d. Sometimes a number of separate data sets from independent experiments were combined in a "merged" Hill plot (e.g. figure 4-18), which consisted simply of a

superposition of the points from individual Hill plots constructed as described above. From such a plot only data points corresponding to a receptor occupancy (θ) between 20% and 80% ($-0.6 \leq \text{logit}\ \theta \leq 0.6$) were included in the subsequent analysis. A curve was fit to the points by a computerized cubic spline algorithm (cubic spline Fortran library subroutine, International Mathematical and Statistical Library; see Reinsch, 1967) that employs polynomials to smooth data in accordance with a built-in statistical criterion (Craven and Wahba, 1979); this method of regression contains no assumptions (other than the continuity of the function and derivatives and smoothness criterion) restricting the basic form or shape of the curve, and thus is appropriate for empirically determining both the shape of the Hill plot and the abscissa and slope at the 50% occupancy point (where logit $\theta = 0$). The slope of the curve at the x-intercept (50% occupancy) was taken as the combined Hill coefficient, and the value of F at this point provided a merged estimate of K_d. (The IMSL subroutine is unable to provide summary statistics for these binding parameters.)

In order to verify that the binding parameters derived from a least-squares linear regression fit to the points on a Scatchard plot were not biased seriously (since the Scatchard coordinate system not only has appreciable error in the independent variable, B_{sp}, but also unfortunately has correlated error in the independent and dependent variables; for review see Rodbard, 1973) a standard computer program (Helwig and Council, 1979) was used to fit some of the binding isotherms by a weighted, nonlinear regression technique (Marquardt, 1963) using the relatively error-free independent variable S_L (total [^{3}H]ligand). The model to which the data were fit consisted of the sum of one

saturable high-affinity ("specific") component and one nonsaturable ("nonspecific") component, which allowed us to further check the validity of the simple linear Scatchard method by omitting subtraction of the 200X "nonspecific" correction. The binding model was the following:

$$B_T = B_0F/(K_d + F) + C_1B_0F. \qquad (4\text{-}6)$$

The actual regression equation (which is rather lengthy) was found by substituting $F = S_L - B_T$ into equation (4-6) and then solving the resulting expression for B_T explicitly. This regression equation (not shown) thus does not contain F; it accepts B_T and S_L as pairs of input variables and then regresses B_T on the relatively error-free variable S_L, fitting the adjustable binding parameters K_d, B_0, and C_1 (the asymptotic "sink" of nonspecific binding). Each input data point was weighted as $1/B_T^2$, consistent with the assumption that the coefficient of variation or percentage error in the measurement of B_T is constant over the range of B_T. The resulting complete 3-parameter regression model was then plotted in the Scatchard coordinate system for display (e.g., figure 4-14), and each of the two components was also plotted separately.

It would also be desirable to combine data sets from separate isotherms in a merged, weighted nonlinear regression of B_T on the relatively error-free variable S_L in a manner similar to that described above for the simple linear "merged Scatchard" analysis. Each value of B_T would be normalized by the appropriate protein concentration to the units fmole/mg protein; the free (F) and total ligand concentration (S_L)

would remain in molar units (e.g., nM). The regression model (equation 4-6) and the actual regression equation would be exactly the same as described above, except that B_T and B_0 now would be expressed in the units fmole/mg protein. The regression would provide estimates of the parameters B_0, K_d, and C_1; and the results of the merged regression would be plotted for display in the same coordinate system as the "merged Scatchard" plot described above. This analysis would also require the assumption that the parameters to be fit do not vary with the cytosol protein concentration.

Although the absence of a correlation between B_0 (fmole/mg) and cytosol concentration made the above assumption seem reasonable, the variability of the individual estimates of receptor concentration (B_0) again suggested that a better merged regression estimate of K_d would be obtained by performing the nonlinear regression with B_0 as an input variable, instead of using it as a unitary parameter to be estimated. Therefore a 2-parameter, 3-variable regression was performed using exactly the same regression model and equation (again weighted as $1/B_T^2$) as described above, except that for each value of B_T, the corresponding estimate of B_0 from a Scatchard analysis of the individual isotherm containing the input data point was used, in addition to the total ligand concentration (S_L), as a second (unweighted) independent variable. The regression program thus generated estimated values of K_d and C_1 and their "asymptotic" standard errors described above. The resulting complete 2-parameter regression model was plotted for display (e.g., figure 4-16) in the "Scatchard-like" coordinate system in which B_T was replaced by B_T/B_0, the normalized individual value of total bound steroid. The measured nonspecific binding corrections (B_{NS}) were sometimes

incorporated into the above regression schemes based on equation (4-6) by simply setting $C_1 = 0$ and concomitantly replacing B_T with B_{sp}. No attempts were made to fit models containing two or more high-affinity, saturable binding sites, since conventional Scatchard plots offered no evidence of binding site heterogeneity.

In order to further examine binding isotherms for apparent "fine structure" that might be indicative of heterogeneity or apparent cooperativity, but that is not obvious from Scatchard or other simple methods of analysis that assume a single class of noninteracting binding sites, some isotherm data sets were transformed into continous affinity distributions or spectra of equilibrium constants by the approximate method of finite differences described by Hunston (1975) and by Thakur, Munson, Hunston and Rodbard (1980). Because the approximate method of finite differences generates a rather broad spectral line (instead of a narrow spike or δ-function) even from "perfect" data derived from models containing only a single class of noncooperative receptors, these transformations of the experimental values of total binding (B_T) were then compared with spectral transformations ("comparison spectra" representating a single class of saturable, noninteracting binding sites) of binding data (B_T) "predicted" by the 3-parameter nonlinear regression model (described above) derived from the same set of experimental data used to generate the "unconstrained" spectrum to be analyzed. Thus, the comparison spectrum consists of a single broad "line" having the minimum line width that can be "resolved" by the finite differences method. Spectra A and B of figure 4-22 are representative, respectively, of the comparison and unconstrained spectra generated by transformation of the data from a single binding

isotherm. Both spectra of such a pair were generated by applying the transformation to smoothed binding functions expressed in the log B_T - log F coordinate system. To generate the comparison spectrum (A) the previously-fit 3-parameter regression equation was transformed to the log B_T - log F coordinate system before use of the finite differences algorithm; thus, the binding data for the comparison spectrum were "smoothed" by force-fit to a specific model containing only one saturable binding site. The unconstrained spectrum (B) was produced by smoothing the experimental B_T data in the same coordinate system with the cubic spline subroutine before transformation to the affinity distribution. Thus, to produce this spectrum the data were not force-fit to any specific biophysical model such as equation (4-6). For display the spectra were normalized by the total receptor concentration (B_0) estimated by the 3-parameter nonlinear regression; thus, the finite difference equation (equation 4 of Thakur et al., 1980, which contains an error in the reference itself) takes the form

$$N(K) = [f_1 - a(f_2 - 2f_1)/(a-1)^2]/2B_0 \log a, \qquad (4\text{-}7)$$

where f_1 and f_2 are as defined in Thakur et al. (1980). The input spacing for the transformation $\Delta \log K = 0.1$, and $\log a = 0.2$. The dimensionality of K is M^{-1}(1/mole); the normalized N(K) itself is dimensionless. The peak of the comparison spectrum is located at ($-\log K_d$) on the abscissa, where K_d is the estimate derived from the 3-parameter nonlinear regression.

Data sets from separate isotherms were also combined to generate pairs of "merged" affinity spectra (e.g., figure 4-24) following the

normalization of each value of B_T by the corresponding estimate of B_0 derived from a Scatchard analysis of the individual isotherm containing the input data point. In this analysis the comparison spectrum (A) is a transformation of normalized total binding data (B_T/B_0) "predicted" by the merged 2-parameter nonlinear regression model (in which B_0 is an input variable, described above) derived from the same set of experimental data. Both spectra of such a pair were generated by applying the transformation to smoothed binding functions expressed in the log (B_T/B_0) - log F coordinate system; the unconstrained spectrum (B) was produced by smoothing the normalized experimental data (B_T/B_0) in the same coordinate system with the cubic spline subroutine before transformation to the affinity distribution as described above. In the merged analysis the values of f_1 and f_2 are dimensionless (since B_T/B_0 is dimensionless) and hence B_0 does not appear in equation (4-7) when it is used for the merged analysis.

The binding selectivity or specificity of the putative glucocorticoid receptors was explored by measuring the affinity of several different unlabeled steroids for the saturable [^{3}H]dexamethasone binding sites. Isotherms were generated with [^{3}H]dexamethasone as described above, first in the absence and then (using the same pool of cytosol) in the presence of a fixed total concentration of the unlabeled competitive inhibitor whose dissociation constant was to be estimated (e.g., figure 4-27). The data resulting from this "Edsall - Wyman" experimental design were analyzed by two different methods. The equilibrium dissociation constant K_{di} for the competitive ligand was first calculated by making the assumption that the concentration of free (unbound) competitor (F_C) was equal to the total concentration present

in the tubes (S_C), and therefore constant over the range of labeled ligand concentration. The effect of a constant concentration of free competitor on a Scatchard plot describing the binding of a labeled ligand to a single class of noncooperative binding sites is given by the Edsall - Wyman equation (e.g., Cantor and Schimmel, 1980), which can be rearranged to the convenient Scatchard form given by equation (3-3) and restated here as

$$B_L/F_L = -(B_L - B_0)/K_d(1 + F_C/K_{di}), \qquad (4\text{-}8)$$

where the subscript L refers to [^{3}H]dexamethasone. Thus, the Edsall - Wyman equation predicts simply that the slope of the Scatchard plot will be reduced by the factor $1/(1 + F_C/K_{di})$ in the presence of a constant concentration of free inhibitor F_C; the estimate of K_{di} was calculated directly from the slopes (estimated by linear regression) of the two Scatchard plots and the total concentration of the inhibitor. However, since the high-affinity competing steroids were not present in great excess over the total concentration of binding sites (B_0), the approximation underlying use of the Edsall - Wyman equation to calculate K_{di} had to be questioned (see chapter III). If the competitive inhibitor is not present in considerable excess over B_0 this approximation will not be valid, the resulting Scatchard plot will be curved (which may not be apparent by visual inspection of the points), and the error in the calculated K_{di} may be substantial.

For this reason a simple and mathematically correct procedure that does not require this assumption was also used to calculate the K_{di} values for the competing steroids. At equilibrium the mass action rate

equations for the binding of the labelled ligand and the competing steroid may be written as

$$(B_0 - B_L - B_C)F_L = K_d B_L \text{ and} \qquad (4\text{-}9)$$

$$(B_0 - B_L - B_C)F_C = K_{di} B_C, \qquad (4\text{-}10)$$

where subscripts L and C refer, respectively, to the labeled and competing ligands. This suggests immediately the following Scatchard-like "free receptor" plot (e.g., figure 4-27) described by the equation

$$B_C/F_C = (B_0 - B_L - B_C)/K_{di}, \qquad (4\text{-}11)$$

where the bound/free ratio now refers to the competing ligand C. Since B_0 and K_d had already been determined from the initial Scatchard plot constructed in the absence of the competitive inhibitor, equation (4-9) was used to calculate the abscissa [$(B_0 - B_L - B_C)$, free binding sites] of the "free receptor" plot described by equation (4-11). The ordinate, B_C/F_C, was calculated with the aid of equation (4-9) in combination with the simple identity $F_C = S_C - B_C$ (i.e., the "nonspecific" binding of competitor C was neglected). A line (which theoretically should satisfy equation 4-11 and pass through the origin) was fit by 2-parameter least-squares linear regression and thus for statistical reasons was not constrained by the analysis to intersect the origin. Since the competition "free receptor" plot estimates only one binding parameter (K_{di}), data from independent experiments employing different cytosol

concentrations and different concentrations of the competing steroid were also merged directly for analysis without further scaling or modification; in such a procedure the nonlinear regression program was again used to generate a standard error (s.e.m.) for the resulting estimate of K_{di}, the reciprocal of the slope of the "free receptor" competition plot. Values of K_{di} calculated by the two different methods were compared.

Results

Efficiency and Linearity of the DEAE Filter Assay

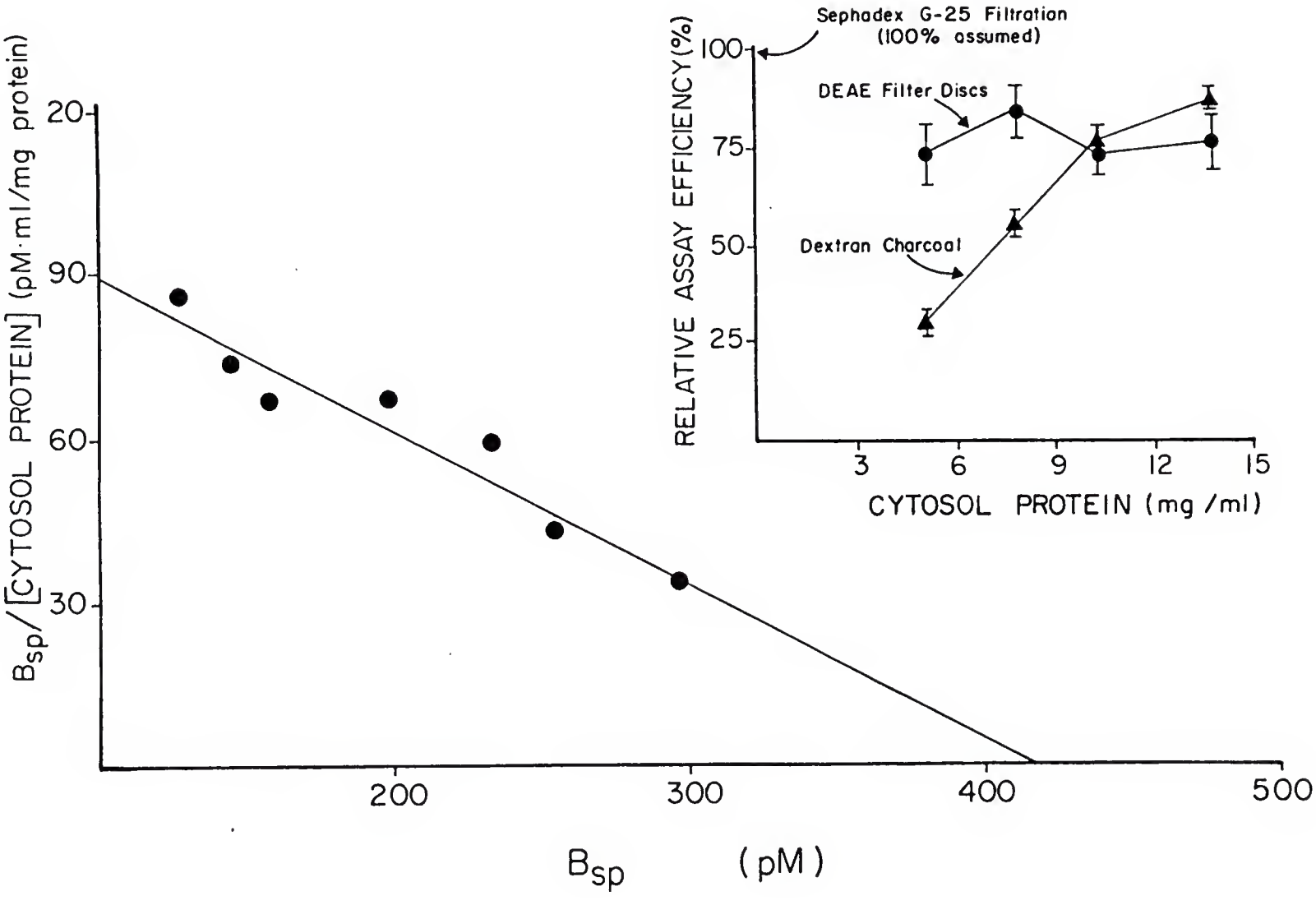
The efficiency of an assay refers to the probability that a specific hormone-receptor complex will be detected by the assay, i.e., the probability that the complex will remain bound to the filter paper and that the [^{3}H]steroid will not dissociate during washing of the free steroid from the filter (Yarus and Berg, 1970). The efficiency of the DEAE filter assay was determined both by titration of a fixed low concentration of [^{3}H]dexamethasone with receptors in increasing concentrations of cytosol protein (suggested by Santi et al., 1973) and by comparison with a simple gel filtration assay whose efficiency was assumed to be 100%; figure 4-1 depicts both methods. In the first method concentrated cytosol was diluted with buffer A to a series of lower concentrations (1:3/4:1/2:1/3:1/4:...1/8). Pairs of samples (250 µl each tube) at each protein concentration were incubated at 2°C for 24 h with a low concentration (approximately 5×10^{-10}M) of [^{3}H]dexamethasone and (for one of each sample pair) a 200-fold excess of unlabeled dexamethasone. The tubes were assayed in triplicate by the DEAE filter assay, and the resulting measured values of specific binding

(B_{sp}) were extrapolated (in the Scatchard-like coordinate system of figure 4-1, which assumes that receptor occupancy is low) by linear regression to infinite protein (and hence receptor) concentration. The ratio of the projected x-intercept of the plot to the concentration of [^{3}H]dexamethasone actually present in the incubation provided an estimate of the assay efficiency; this procedure assumed that the functional receptor concentration was linear with protein concentration (see below and figure 4-26) and that the assay itself was linear with protein concentration (i.e., had constant efficiency) within the range of protein concentration employed (see below). The method yielded an efficiency estimate of 76% ± 2% (n=3); at all protein concentrations the nonspecific corrections were very low and had little effect on the final estimate of the efficiency.

The efficiency of the filter assay was also determined by direct comparison with gel filtration and dextran-charcoal (DCC) assays performed with the same cytosol samples at diffferent protein concentrations (inset to figure 1). Following incubation with 2×10^{-8} M [^{3}H]dexamethasone (with and without unlabeled dexamethasone) pairs of cytosol samples were assayed (as 50 µl triplicates) for specific binding (B_{sp}) by the DEAE assay, by filtration on Sephadex G-25 minicolumns, or by adsorption of free steroid onto dextran-coated charcoal as described in Methods. In agreement with the efficiency determined by titration with increasingly concentrated cytosol samples, the DEAE filter assay measured 76 ± 3% (n=3 experiments) of the specific binding detected by the G-25 minicolumn assay (assumed arbitrarily to possess 100% efficiency); this ratio was not dependent upon protein concentration.

Fig. 4-1. Determination of the efficiency of the DEAE filter assay by titration of a fixed concentration of [^{3}H]dexamethasone with glucocorticoid receptors in increasing concentrations of cytosol protein.

The ratio of the projected x-intercept of the "Scatchard-like" plot to the concentration of [^{3}H]dexamethasone actually present in the incubation provides an estimate of the assay efficiency. The depicted protein concentrations ranged from 1.5 to 8.9 mg/ml, and the constant [^{3}H]dexamethasone concentration was 0.58 nM. For the particular experiment shown above the efficiency estimate was 72%. Inset: determination of the efficiency (relative to the Sephadex G-25 filtration assay) of the DEAE filter assay and of the dextran-coated charcoal assay at different cytosol protein concentrations. The relative efficiencies plotted above are $\bar{x} \pm$ s.e.m. for 3 independent determinations at a constant [^{3}H]dexamethasone concentration (2×10^{-8} M). The DEAE assay efficiency (●) is not affected by protein concentration in the range depicted above, but that of the dextran-charcoal assay (▲) is strongly dependent on the cytosol concentration.

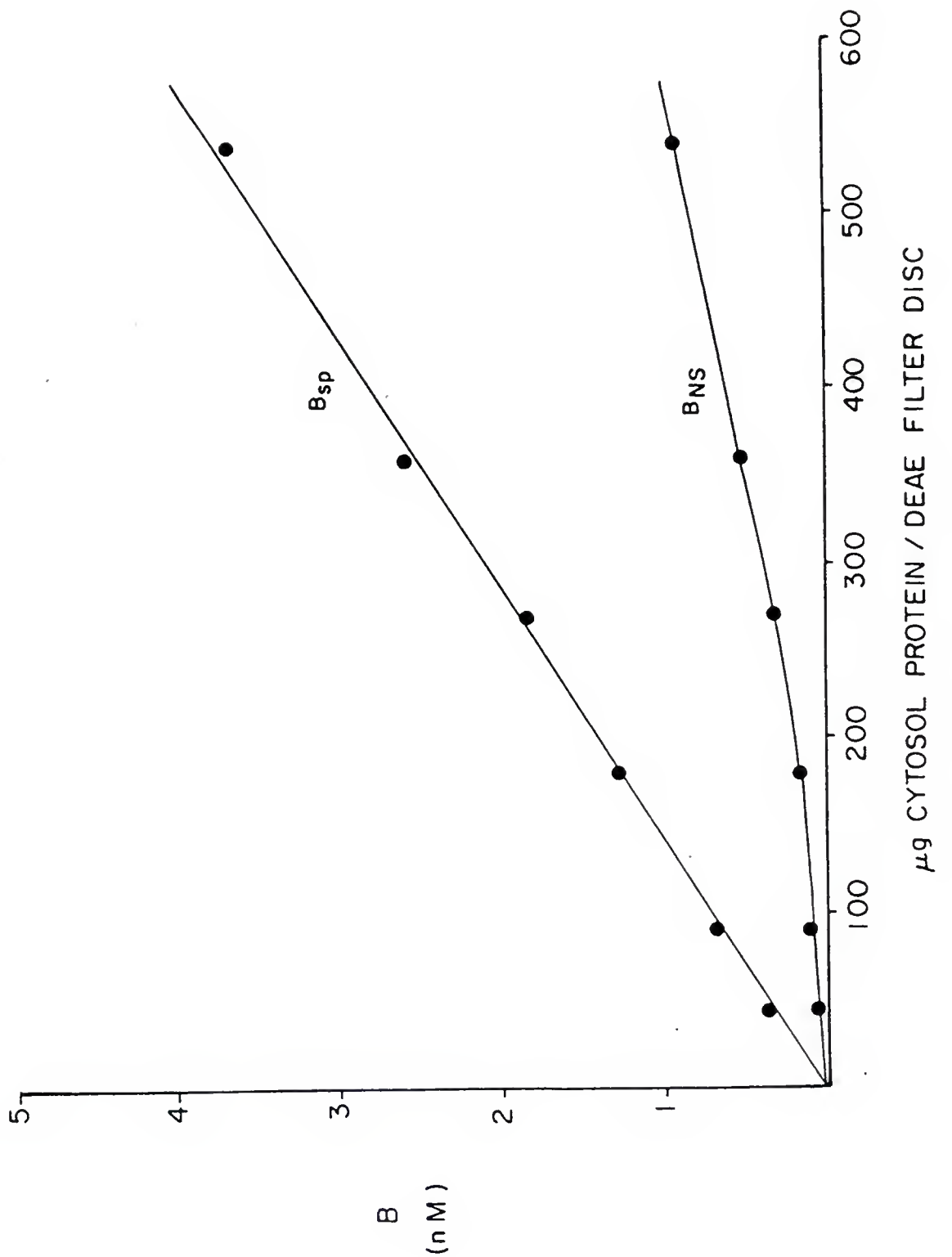

Sephadex G-25 Filtration
(100% assumed)
DEAE Filter Discs
Dextran Charcoal
RELATIVE ASSAY EFFICIENCY(%)
100
75
50
25
3
6
9
12
15
CYTOSOL PROTEIN (mg /ml)
Bsp/[CYTOSOL PROTEIN] (pM·ml/mg protein)
20
90
60
30
200
300
400
500
Bsp (pM)

The efficiency of the charcoal (DCC) assay (85% relative to the gel for filtration column assay at the highest protein concentration, decreasing to approximately 30% at the lowest) was strongly dependent on protein concentration, apparently because the samples were diluted 20:1 by the assay conditions; this reduction of the DCC assay efficiency caused by receptor adsorption to the charcoal at low protein concentrations has often been observed (e.g., Katzenellenbogen, Johnson and Carlson, 1973). Thus, for all subsequent determinations of affinity and binding capacity the efficiency of the DEAE filter assay was taken to be 76%; this value is essentially equivalent to the efficiency reported for a similar assay for serum sex steroid binding protein (Mickelson and Petra, 1974) and is somewhat lower than the 86% efficiency reported for hepatoma cell (HTC) glucocorticoid receptors (Santi et al., 1973). The efficiency of such ion-exchange filter assays depends upon the macromolecular species to be measured and upon the pH, ionic strength, and composition of the buffer.

Figure 4-2 displays the linearity of the DEAE filter assay explored as a function of the amount of cytosol protein applied to the filters. Pairs of cytosol samples equilibrated with 2×10^{-8} M [^{3}H]dexamethasone and (for one of each sample pair) a 200-fold excess of unlabeled dexamethasone were diluted rapidly to the desired protein concentration, and 50 µl samples were assayed in triplicate for the determination of specific binding (B_{sp}). The assay was linear in the range explored, which extended from 50 to 600 µg cytosol protein per filter (corresponding to cytosol protein concentrations ranging from 1-12 mg/ml); the similar assay described by Santi et al. (1973) is linear in the range extending from 0-4000 µg protein/filter (corresponding to

Fig. 4-2. Linearity of the retention of [^{3}H]dexamethasone-receptor complexes by DEAE filters as a function of the amount of cytosol protein applied.

Samples incubated with 2 x 10^{-8} M dexamethasone were diluted in rapid serial fashion and 50 µl aliquots were filtered in triplicate. Upper curve: specifically bound steroid. Lower curve: nonspecifically bound steroid defined by incubation of the cytosol with [^{3}H]dexamethasone and a 200-fold excess of unlabeled steroid before dilution and filtration. The undiluted protein concentration was 10.8 mg/ml, and the receptor concentration (B_0) was 357 fmole/mg protein. The experiment was repeated 3 times with the same resulting linearity of the specific binding.


B
(nM)
5
4
3
2
1
100
200
300
400
500
600
μg CYTOSOL PROTEIN / DEAE FILTER DISC
B_{sp}
B_{NS}

cytosol protein concentrations ranging from 0-80 mg/ml). The experiment was repeated (n=3) with the same result.

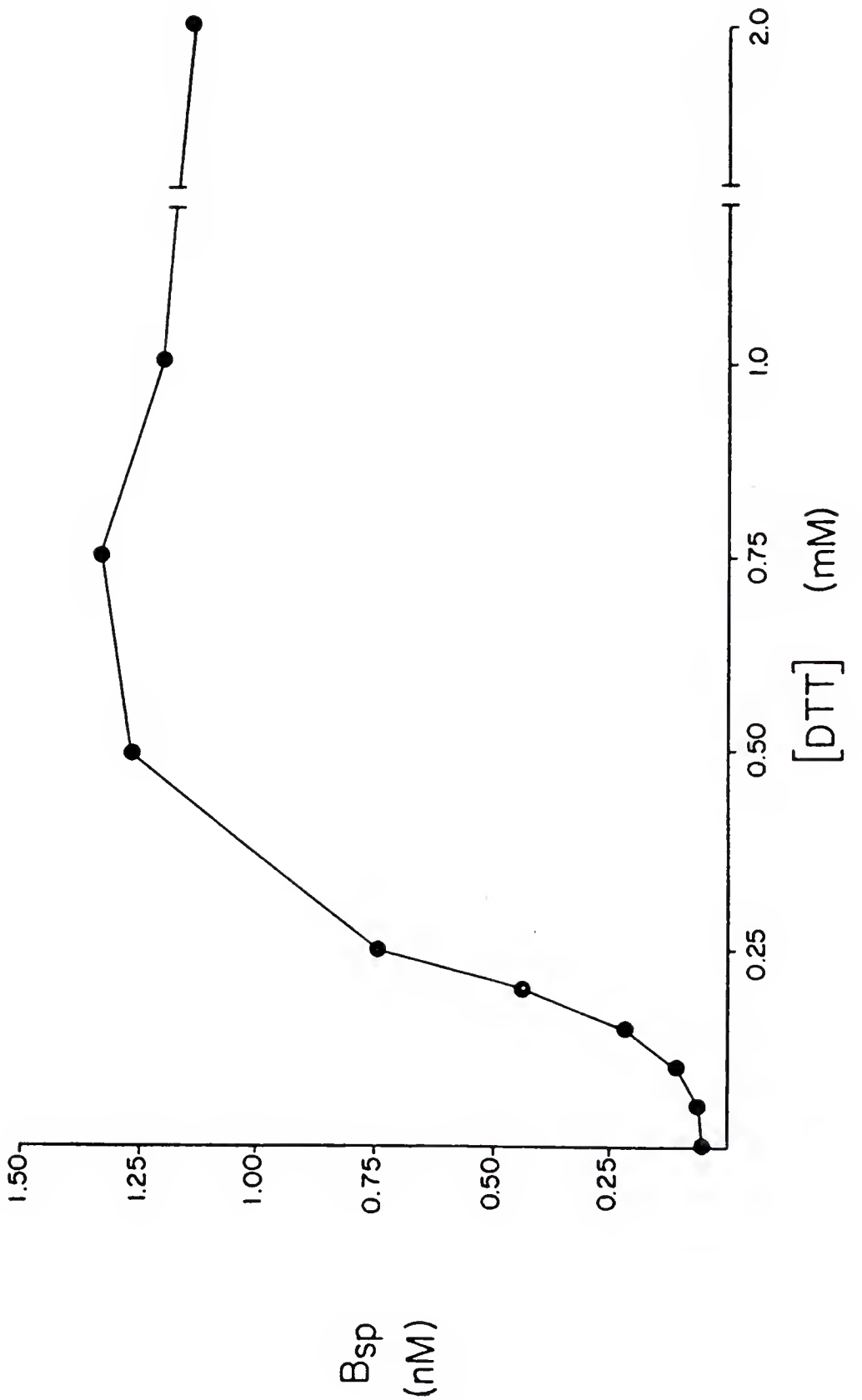
Effects of Medium Composition and Temperature on In Vitro Receptor Stability

In order to minimize the loss of functional binding sites during cytosol preparation and lengthy incubations, we examined the effect of varying the pH, the concentration of the sulfhydryl-protective reagent dithiothreitol (DTT), and the concentration of sodium molybdate (Na_2MoO_4) on the specific [^{3}H]dexamethasone binding capacity (B_{sp}) assayed at high receptor occupancy (near saturation); the other components of buffer A were not varied. Specific [^{3}H]dexamethasone binding capacity remained constant within the pH range from 7.0 to 8.2 that was explored (not shown), and buffer adjusted to pH 7.6 was used for all further experiments. (Depending upon the tissue:buffer ratio, the pH of cytosol can be .1-.2 pH units lower than the pH of the homogenization buffer.)

The strong effect of dithiothreitol (DTT) on the in vitro stability of glucocorticoid receptor steroid binding sites is shown in figure 4-3. Cytosol was prepared in the buffer A (without DTT), and pairs of samples were adjusted to the different DTT concentrations with a concentrated DTT stock solution (containing the other buffer A ingredients) and additional buffer A. Following 12 hours of incubation without steroid ("aging") at 2 C the samples were incubated for 12 h at 2 C with 2 x 10^{-8} M [^{3}H]dexamethasone and excess "cold" dexamethasone (one of each pair). 50 µl aliquots were then assayed in triplicate with the DEAE filter assay for the determination of specific binding (B_{sp}). Essentially all functional binding sites were lost in the absence of

Fig. 4-3. Effect of dithiothreitol (DTT) concentration on the stability of mouse brain glucocorticoid receptors.

Cytosol was prepared in buffer A (without DTT), and pairs of samples were adjusted to the different DTT concentrations with a concentrated DTT stock solution (containing the other buffer A ingredients) and additional buffer A. Following 12 hours of "aging" without steroid at 2°C the samples were incubated for 12 h at 2°C with 2×10^{-8}M [^{3}H]dexamethasone and excess "cold" dexamethasone (one of each pair). 50 µl aliquots were assayed in triplicate with the DEAE filter method for the determination of specific binding. The final cytosol protein concentration was 4.1 mg/ml. The "plateau" receptor concentration (mean of determinations at 4 highest DTT concentrations) was 300 fmole/mg cytosol protein.


1.50
1.25
1.00
0.75
0.50
0.25
Bsp
(nM)
0.25
0.50
0.75
1.0
2.0
[DTT] (mM)

DTT, and the measured plateau receptor concentration was achieved with 0.5-0.75 mM DTT; subsequent experiments were performed with 2 mM DTT to prevent the oxidation of sulfhydryl groups that affect the conformation of the steroid binding sites. Other investigations of glucocorticoid receptors from a variety of tissues have shown that the loss of functional binding sites in the absence of DTT occurs during the period of cytosol "aging" without steroid; formation of the glucocorticoid-receptor complex stabilizes the receptor sulfhydryl groups and prevents their oxidation and the subsequent further loss of functional binding sites in the absence of DTT (e.g., Granberg and Ballard, 1977).

The large effect of molybdate ($MoO_4^{=}$) ion on the in vitro stability of glucocorticoid binding sites is shown in figure 4-4. Cytosol was prepared in buffer A (without Na_2MoO_4), and pairs of samples were adjusted to the different Na_2MoO_4 concentrations with a concentrated Na_2MoO_4 stock solution (containing the other buffer A ingredients) and additional buffer A. Following 12 hours of "aging" without steroid at 2°C or at 22°C the sample pairs were incubated for 12 h at 2°C with 2×10^{-8} M [^{3}H]dexamethasone and excess unlabeled steroid (one of each pair). 50 µl aliquots were assayed in triplicate with the DEAE filter assay for the determination of specific binding (B_{sp}). Cytosol incubated for 12 h at 2°C without steroid required only 1-2 mM Na_2MoO_4 (perhaps less) for prevention of the loss of functional binding sites ("decapacitation"). In contrast, essentially all sites were lost at room temperature in the absence of molybdate, and attainment of the measured plateau receptor concentration required approximately 8 mM Na_2MoO_4 for full protection from heat inactivation. Figure 4-4 also

shows (inset) that the 10 mM Na_2MoO_4 contained in buffer A is fully protective of unoccupied binding sites during low-temperature incubations lasting as long as 24 h, a condition that is necessary for the construction of meaningful binding isotherms with ligands that require long incubations for the attainment of equilibrium. The heat-accelerated inactivation of binding sites ("decapitation") and the protective effect of the concentration of molybdate chosen for buffer A (10 mM) were studied further in relation to the time and temperature of the "aging" incubations (without steroid). Cytosol was prepared in buffer A (figure 4-5, upper panel) or buffer A without molybdate (lower panel). Following a variable period of "aging" without steroid at 2°C, 12°C, 22°C or 32°C the concentration of functional binding sites remaining in the cytosol was assayed as described above. In the absence of molybdate the unoccupied receptor pool was inactivated at 2°C with a half-life ($t_{\frac{1}{2}}$) of about 10 hours; at 12°C the $t_{\frac{1}{2}}$ decreased to approximately 1 h, and at room temperature (22°C) and above the loss of sites (decapitation) was very rapid. (In the experiment shown in figure 4-5 the rate of decapacitation at 2°C in the absence of molybdate was slightly faster than the rate suggested by the initial point on the 2°C curve of figure 4-4). Consistent with the results presented in figure 4-4 and described above, the molybdate concentration of buffer A (10 mM) was fully protective for at least 10 h, except at the highest, 32°C "aging" temperature. (All known steroid receptors are unstable at such high, relatively "physiological" temperatures under all in vitro conditions that have yet been explored.)

The protective effect of molybdate on unoccupied mouse brain glucocorticoid binding sites indicated by the data of figures 4-4 and 4-5 is

Fig. 4-4. Effect of molybdate concentration on the stability of mouse brain glucocorticoid receptors.

Cytosol was prepared in buffer A (without Na_2MoO_4), and pairs of samples were adjusted to the different Na_2MoO_4 concentrations with a concentrated Na_2MoO_4 stock solution (containing the other buffer A ingredients) and additional buffer A. Following 12 hours of "aging" without steroid at 2°C ( ) or at 22°C ( ) the samples were incubated for 12 h at 2°C with 2 x 10^{-8}M [^{3}H]dexamethasone and excess unlabeled steroid (one of each pair). 50 µl aliquots were assayed in triplicate by the DEAE filter method for the determination of specific binding. Because two completely independent experiments were depicted, each value of specific binding (B_{sp}) has been normalized by the "plateau" level of B_{sp} for the particular experiment (B_0^*, the mean of the determinations at the 4 highest molybdate concentrations). The final cytosol protein concentrations were 5.6 mg/ml for the 2°C experiment and 4.7 mg/ml for the 22°C experiment. The "plateau" receptor concentrations were 240 fmole/mg protein (2°C) and 380 fmole/mg protein (22°C). Inset: stability of unoccupied mouse brain glucocorticoid receptors at 2°C in buffer A (containing 10mM Na_2MoO_4). Cytosol was prepared in buffer A and assayed for specific binding as described above after the variable "aging" period. The origin (0) of the time axis corresponds to the end of the 90 min cytosol preparation period when the first samples were labeled with [^{3}H]dexamethasone. The values displayed are $\bar{x}$ ± s.e.m. for 3 independent experiments. Cytosol protein concentration was 7 mg/ml, and the mean receptor concentration was 330 fmole/mg cytosol protein.

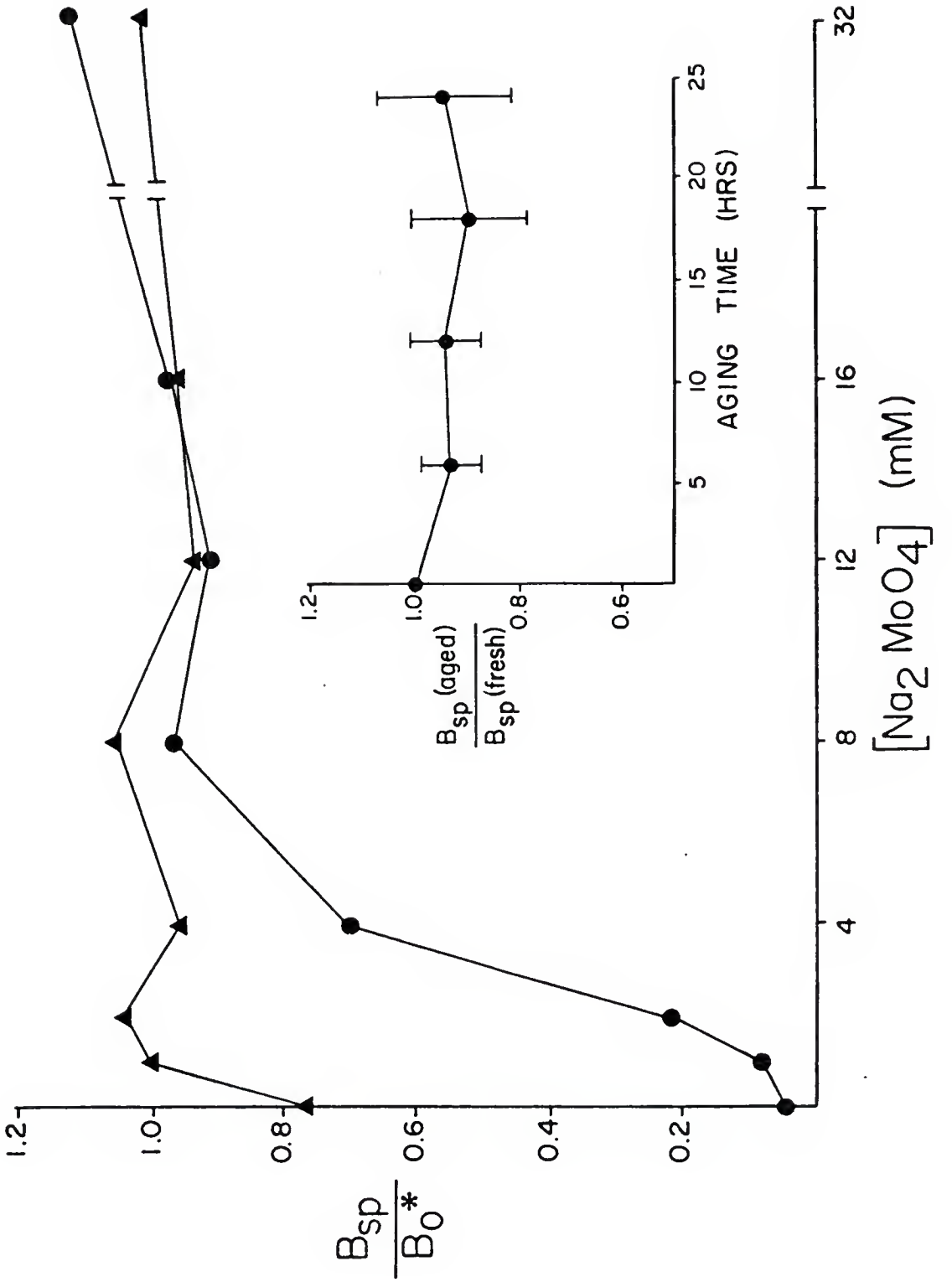
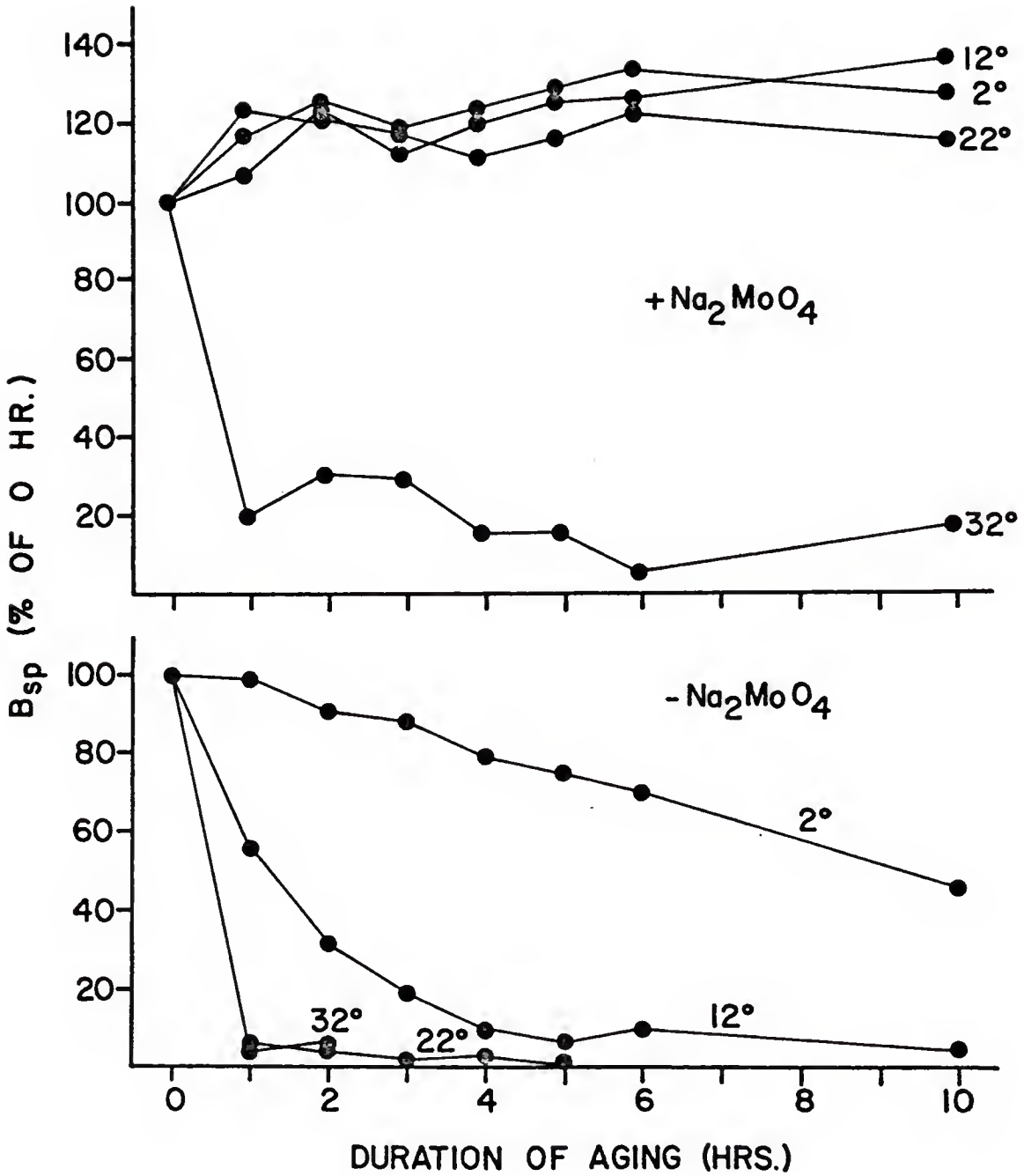

1.2
1.0
0.8
0.6
0.4
0.2
$\frac{B_{sp}}{B_o^*}$
4
8
12
16
32
$[Na_2MoO_4]$ (mM)
1.2
1.0
0.8
0.6
$\frac{B_{sp}(aged)}{B_{sp}(fresh)}$
5
10
15
20
25
AGING TIME (HRS)

Fig. 4-5. Inactivation of mouse brain glucocorticoid receptor binding sites ("decapacitation") as a function of time, temperature, and the presence or absence of 10mM Na_2MoO_4.

Cytosol was prepared in buffer A (upper panel) or buffer A without molybdate (lower panel). Following a variable period of "aging" without steriod at 2°C, 12°C, 22°C or 32°C the pairs of samples were incubated for 6 h at 2°C with 1 x 10^{-8} M [^{3}H]dexamethasone and excess (200X) unlabeled steroid (one of each pair). 50 µl aliquots were assayed in triplicate with the DEAE filter assay for the determination of specific binding. Because experiments using two independent pools of cytosol are depicted, each value of specific binding (B_{sp}) has been normalized by the initial measured value of B_{sp} for the particular pool of cytosol. The origin of the time axis corresponds to the end of the 90 min cytosol preparation period. Cytosol protein concentrations were 3.1 mg/ml (upper panel) and 3.2 mg/ml (lower panel); initial values of B_{sp} were 270 fmole/mg protein (upper panel) and 310 fmole/mg protein (lower panel). The molybdate concentration of buffer A (10 mM) is fully protective for at least 10 h, except at the highest aging temperature (32°C).


140
120
100
80
60
40
20
12°
2°
22°
+Na2MoO4
32°
Bsp (% OF 0 HR.)
100
80
60
40
20
-Na2MoO4
2°
32°
22°
12°
0
2
4
6
8
10
DURATION OF AGING (HRS.)

consistent with reports of similar results from studies with glucocorticoid receptors from a variety of other sources (e.g., Nielsen, Sando and Pratt, 1977; Leach, Dahmer, Hammond, Sando and Pratt, 1979); actual rates of heat-induced glucocorticoid receptor decapacitation in the absence of molybdate vary with tissue source and cell type as well as with cytosol concentration, possibly because different preparations possess different concentrations of relevant endogenous factors in addition to the receptors themselves.

The molybdate concentration of buffer A (10 mM) has also been reported to completely block the activation (nucleophilic transformation) of glucocorticoid receptors from a variety of tissues (e.g., Leach et al., 1979; Dahmer, Quasney, Bissen and Pratt, 1981), permitting the measurement of kinetic and equilibrium binding parameters characteristic of a single homogeneous pool of receptors (i.e., non-activated, albeit probably influenced by the presence of molybdate). Furthermore, the results of DEAE minicolumn chromatography performed as described in Methods suggested that the activation of mouse brain glucocorticoid receptors incubated in cold buffer A was negligible. Consistent with similar results from studies of nonactivated receptors from other sources (e.g., Vedeckis, 1981), a single peak of bound [^{3}H]TA was eluted at the ionic strength corresponding to buffer A + 160 mM KCl (not shown), indicating that the entire receptor population remained in the more acidic, nonactivated form. The mechanisms by which molybdate exerts its protective effect on steroid binding sites and prevents nucleophilic receptor transformation (activation) are, however, mysterious; these are currently subjects of intense investigation. Current speculations include both direct

molybdate-receptor interactions and indirect influences mediated by effects of molybdate on other endogenous modulators, such as the enzymes of a phosphorylation-dephosphorylation cycle (e.g., Grody, Schrader and O'Malley, 1982, for review).

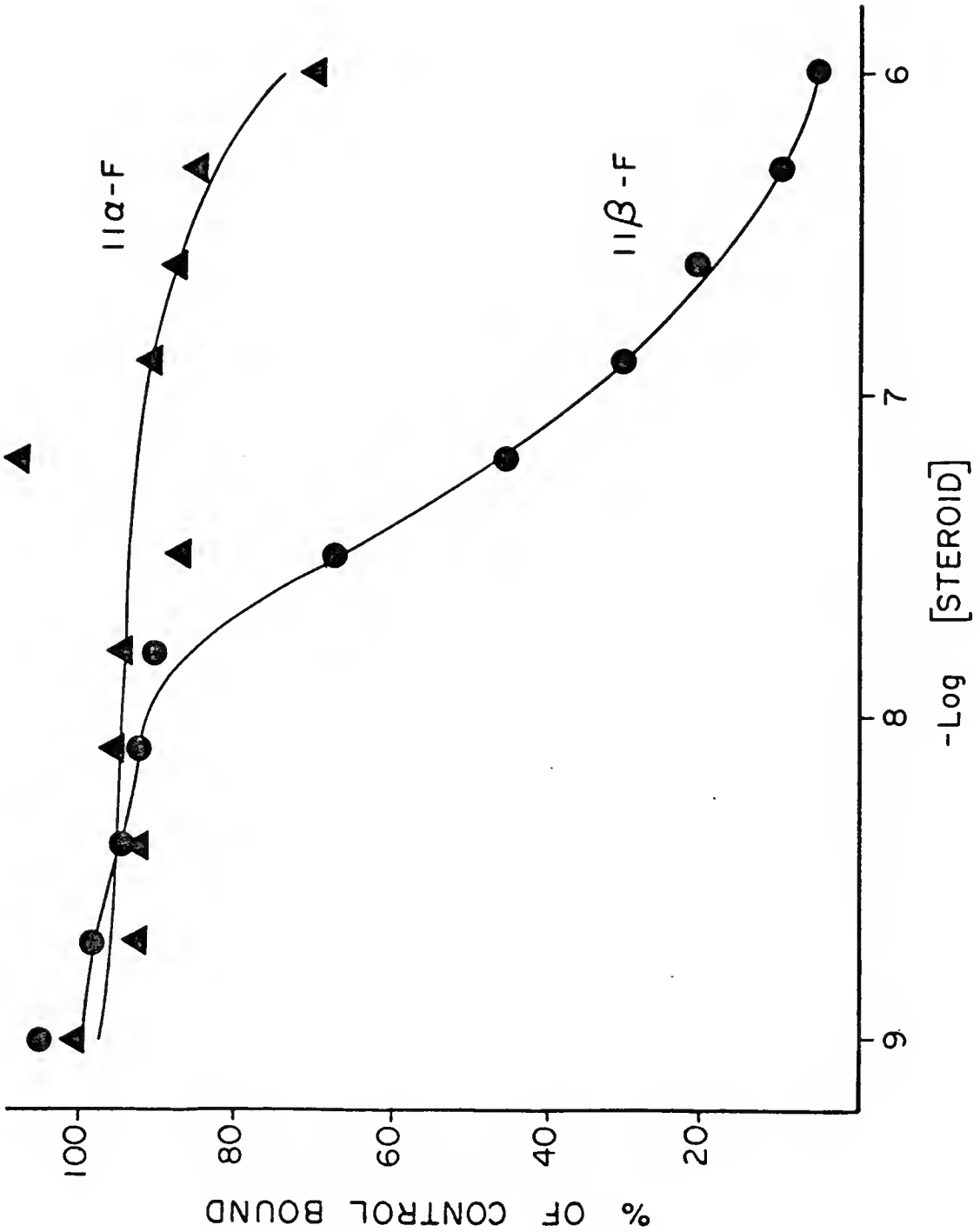
Equilibrium Binding Characteristics of the Mouse Brain Glucocorticoid Receptors

In addition to the necessary demonstration of a saturable population of high-affinity binding sites having specificity for glucocorticoid agonists and antagonists, some important aspects of the characterization of the receptors are: 1) confirmation that the binding sites are highly selective for a specific molecular shape (sterospecificity), 2) the determination of the subcellular distribution of the binding sites, and 3) confirmation of the chemical identity of the ligand actually bound in the hormone-receptor complex under investigation.

Figure 4-6 reveals a high degree of stereospecificity in the glucocorticoid-receptor interaction that was demonstrated by comparing the inhibitory potencies of the diastereomers cortisol (11β-F) and epicortisol (11α-F, a non-glucocorticoid) as competitive inhibitors of the binding of [^{3}H]dexamethasone. Cytosol was prepared in buffer A, and pairs of samples were adjusted to the indicated concentrations of cortisol or epicortisol. The pairs of samples were then incubated for 16 h at 2°C with 6 nM [^{3}H]dexamethasone and 200-fold excess cold dexamethasone (one of each pair). 50 µl aliquots were assayed in triplicate with the DEAE filter assay for the determination of specific binding. As expected, only cortisol (the 11β-OH compound) was an effective competitor for the [^{3}H]dexamethasone binding sites, indicating

Fig. 4-6. Stereospecificity of steroid binding to mouse brain glucocorticoid receptors labelled with [^{3}H]dexamethasone.

Cytosol was prepared in buffer A, and pairs of samples were adjusted to the indicated concentrations of the diastereomers cortisol (11β-F, ●) or epicortisol (11α-F, ▲). The pairs of samples were then incubated for 16 h at 2°C with 6 nM [^{3}H]dexamethasone and excess (200X) cold dexamethasone (one of each pair). 50 µl aliquots were assayed in triplicate with the DEAE filter assay for the determination of specific binding. The values of specific binding have been normalized by the level of specific binding measured in the absence of competing cortisol or epicortisol (1.4 nM or 230 fmole/mg protein). Cytosol protein concentration was 5.9 mg/ml. Only the 11β-OH compound was an effective competitor for the [^{3}H]dexamethasone binding sites.


11α-F
11β-F
% OF CONTROL BOUND
100
80
60
40
20
9
8
7
6
-Log [STEROID]

the importance of the ligand shape determined by the spatial arrangement of the functional groups on the steroid nucleus; the 11β-OH is an absolute requirement for glucocorticoid activity.

Subcellular fractionation (in buffer A, a hypertonic medium) into nuclear, crude mitochondrial, microsomal and cytosol components revealed that essentially all high-affinity [^{3}H]dexamethasone binding sites (assayed at 2 x 10^{-8} M [^{3}H]dexamethasone) were released into the high-speed supernatant following homogenization in buffer A; the subcellular distribution of unoccupied binding sites in vivo is not known.

Dexamethasone does not undergo metabolic transformation; the extremely remote possibility that the endogenous corticosterone requires metabolic activation before binding to the receptors was eliminated by checking the chemical identity of radioactivity recovered from the DEAE filters following the assay of cytosol labeled with [^{3}H]corticosterone. All radioactivity recovered from the filters was indistinguishable from authentic [^{3}H]corticosterone by the LH-20 chromatographic separation described by Sippell et al. (1975).

Equilibrium isotherms constructed with increasing concentrations of [^{3}H]dexamethasone (e.g., figure 4-7), [^{3}H]TA (not shown), and [^{3}H]corticosterone (e.g., figure 4-8) revealed saturable populations of high-affinity binding sites. The apparent affinities for the three ligands were, however, quite different; and the concentration of binding sites for [^{3}H]corticosterone was significantly greater than the approximately equal concentrations of [^{3}H]dexamethasone and [^{3}H]TA binding sites. The saturation isotherms all approximated rectangular hyperbolas, suggesting that each ligand was apparently interacting with a single population of saturable, high-affinity receptors. No evidence

Fig. 4-7. Representative equilibrium isotherm for the binding of [^{3}H]dexamethasone to glucocorticoid receptors in perfused mouse brain cytosol.

Cytosol was prepared in buffer A, and 15 pairs of samples (250 µl) were incubated for 24 h at 2°C with increasing concentrations of [^{3}H]dexamethasone and, for one of each sample pair, a 200-fold excess of unlabeled steroid. 50 µl aliquots were than assayed in triplicate by the DEAE filter method for the determination of total binding (B_T, ■) and nonspecific binding (B_{NS}, ▲). Specifically bound steriod (B_{sp}, ●) was determined by substracting B_{NS} from B_T. The concentration of free steroid (F) is plotted on the x-axis. For the specific experiment depicted above the cytosol protein concentration was 6.6 mg/ml and the total [^{3}H]dexamethasone concentration ranged from 0.06 to 420 nM (the two determinations at total [^{3}H]dexamethasone concentrations above 25 nM are not shown).

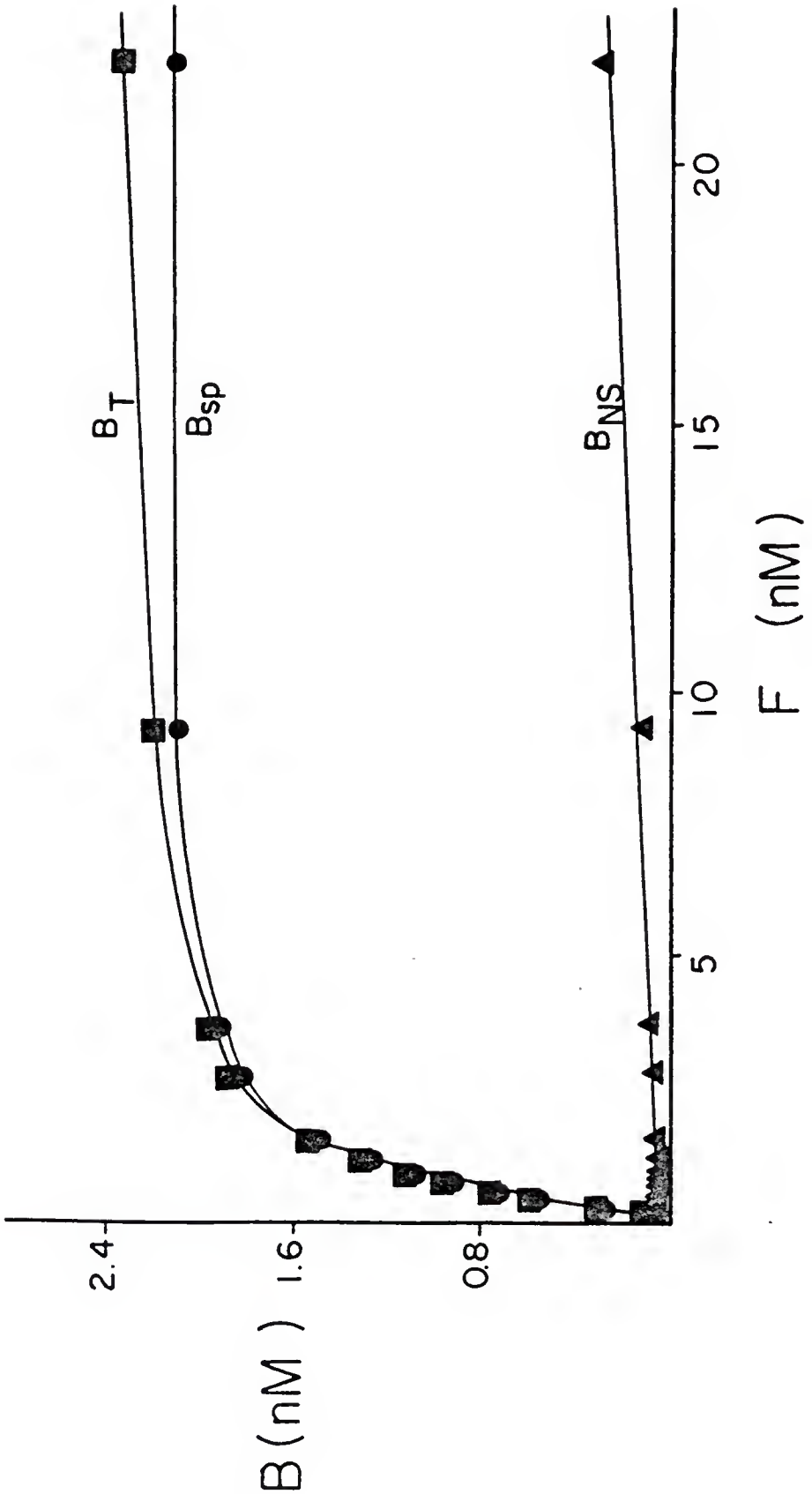
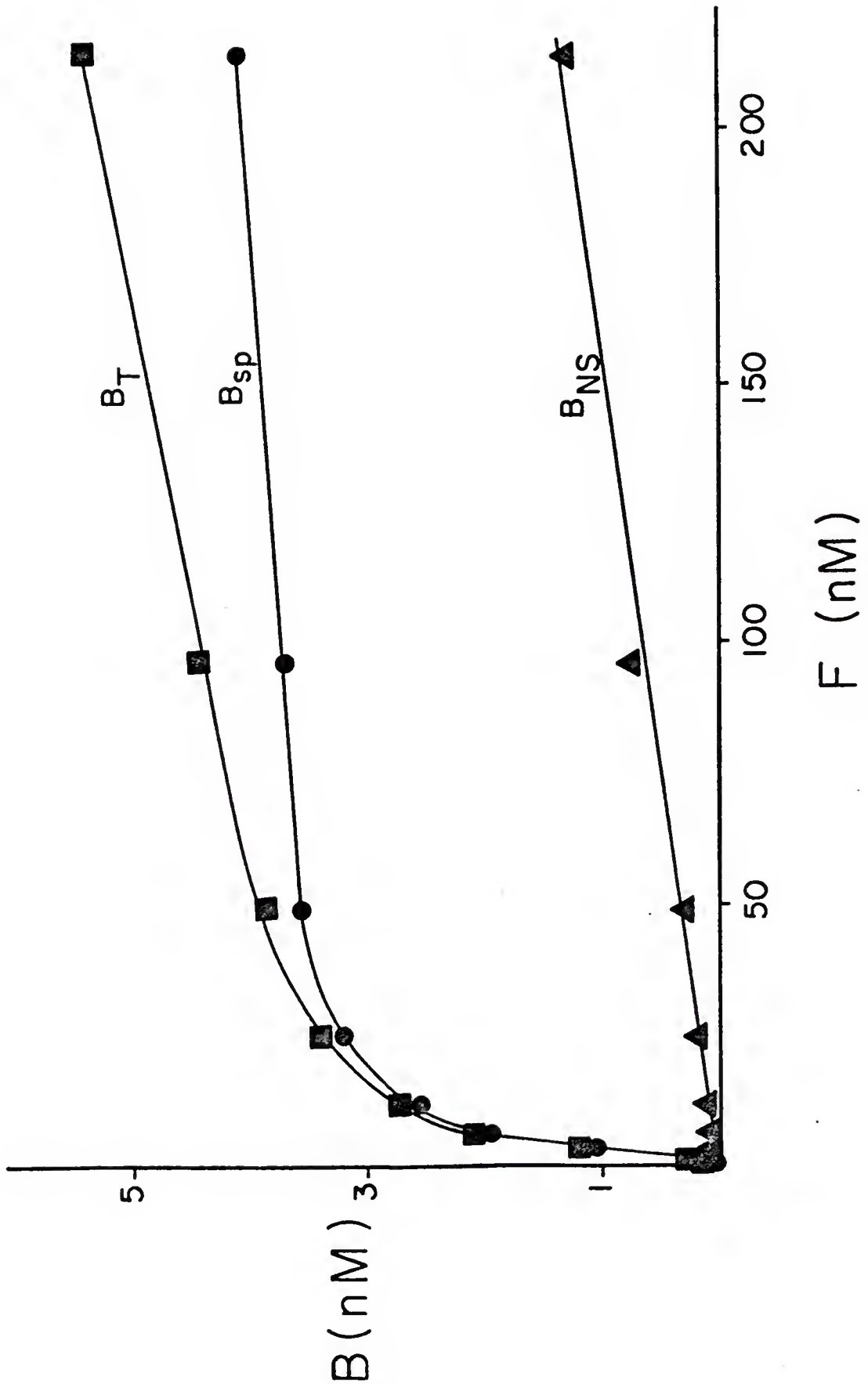

B(nM)
2.4
1.6
0.8
5
10
15
20
F (nM)
B_T
B_{sp}
B_{NS}

Fig. 4-8. Representative equilibrium isotherm for the binding of [^{3}H]corticosterone to the complete ensemble of saturable high-affinity binding sites in perfused mouse brain cytosol.

Cytosol was prepared in buffer A, and pairs of samples (250 µl) were incubated for 16 h at 2°C with increasing concentrations of [^{3}H]corticosterone and, for one of each sample pair, a 200-fold excess of unlabeled corticosterone. 50 µl aliquots were then assayed in triplicate by the DEAE filter method for the determination of total binding (B_T,■) and nonspecific binding (B_{NS},▲). Specifically bound steroid (B_{sp},●) was determined by subtracting B_{NS} from B_T. The concentration of free steroid (F) is plotted on the x-axis. For the specific experiment depicted the cytosol protein concentration was 7.0 mg/ml and the total [^{3}H]corticosterone concentration ranged from 0.3 nM to 2800 nM (one measurement at highest [^{3}H]corticosterone concentration not shown).


B(nM)
5
3
1
50
100
150
200
F (nM)
B_T
B_{sp}
B_{NS}

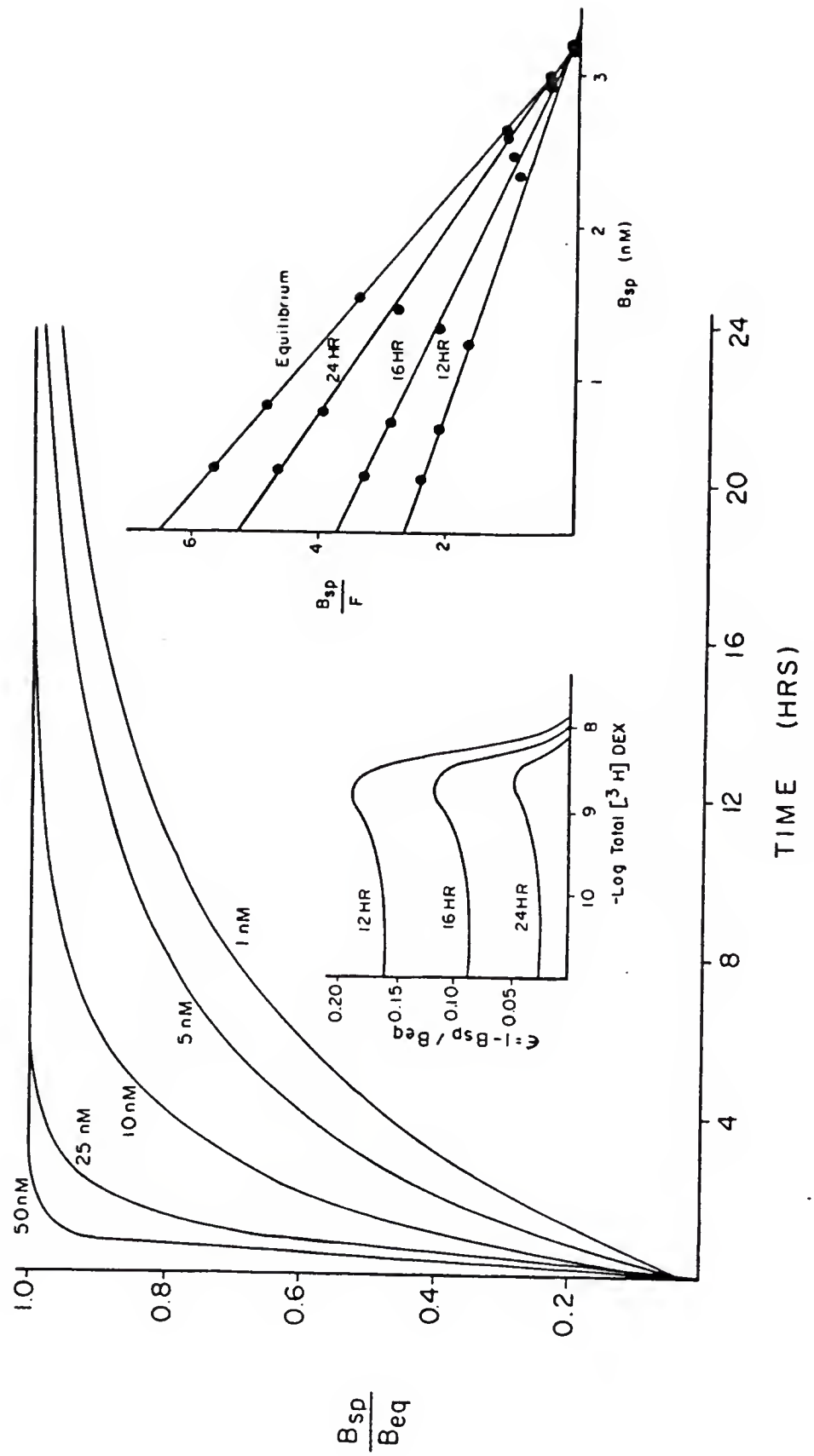
for secondary populations of saturable, lower-affinity binding sites was observed when the [^{3}H]ligand concentrations were increased at least two orders of magnitude above the apparent K_d for the high-affinity sites. The nonspecific binding component (B_{NS}) continued to increase approximately linearly as the [^{3}H]ligand concentrations were increased. For each ligand the signal/noise (B_{sp}/B_{NS}) ratio was excellent when [^{3}H]steroid concentrations did not greatly exceed amounts required for near-saturation. For each ligand the data were superficially compatible with a model consisting of one class of noninteracting, saturable sites and a nonsaturable, approximately linear nonspecific component. The data were analyzed by the transformations (e.g., Scatchard) and regression schemes described above in Methods.

The problem of choosing appropriate incubation times for the isotherm experiments required detailed analysis. Estimates of the time required to reach equilibrium at the low [^{3}H]dexamethasone concentrations were obtained both by direct experimental observations and also indirectly from a theoretical simulation of the rate of approach to equilibrium calculated from the measured values of the [^{3}H]dexamethasone association and dissociation rate constants (to be presented and discussed in chapter VI) and an assumed, "typical" value of the binding site concentration. Disappointingly, the observed and "theoretical" estimates of the required incubation times were not in close agreement. Thus, before presenting the measured equilibrium binding parameters we must digress to discuss the problem of the choice of an adequate incubation time. The information presented in chapter II will serve as background for the following description of the simulation of the time course of [^{3}H]dexamethasone binding.

The simulated approach to equilibrium is depicted in figure 4-9, which presents the fraction of the equilibrium value of specific binding attained as a function of the time of incubation, with the total [^{3}H]dexamethasone concentration shown as the parameter. The calculation required the mean values of the measured [^{3}H]dexamethasone association and dissociation rate constants (described in chapter VI) as inputs to the previously-discussed equations (2-5) and 2-13). The assumed "typical" cytosol binding site concentration (B_0) was 3.2 mM (B_0 ranged from 2.1 to 3.9 mM in the dexamethasone isotherm experiments), and the mean measured values of the association and dissociation rate constants assumed for the calculation were $k_a = 0.69 \times 10^6$ M^{-1} min^{-1} and $k_d = 3.4 \times 10^{-4}$ min^{-1} (see chapter VI). Figure 4-9 also presents (left inset) a simulation of the relative deviation from equilibrium (ϵ) of specific binding (B_{sp}) plotted as a function of the total [^{3}H]-dexamethasone concentration, with incubation time as the indicated parameter. The same rate constants and receptor concentration (listed above) were input to equation (2-28) to generate the relative error (ϵ) curves; for this specific simulation the curves have maxima in the low nanomolar range and actually decrease to approach constant values as the steroid concentration is reduced. Thus, if an incubation time is chosen such that the deviation from equlibrium (relative error ϵ) is reduced to an acceptable level for the samples incubated with 1 nM [^{3}H]dexametha-sone, then samples incubated with even lower concentrations of [^{3}H]dexamethasone may also be used to generate the isotherm, since they will attain at least as close fractional approaches to equilibrium in the given time. Finally, figure 4-9 (right inset) presents the

Fig. 4-9. Simulated approach to equilibrium of specifically bound steroid (for a representative set of incubations of [^{3}H]dexamethasone with glucocorticoid receptors in perfused mouse brain cytosol) as a function of time and total ligand concentration.

The fraction of the final equilibrium value attained is plotted on the ordinate as a function of time after mixing, with total steroid concentration as the indicated parameter. The simulation was performed by using the mean values of the measured steroid association and dissociation rate constants (described in chapter VI) as inputs to equations (2-5) and (2-13). The assumed binding site concentration (B_0) was 3.2 nM, the mean generated by 16 individual isotherms whose values of B_0 ranged from 2.1 to 3.9 nM. The mean measured values of the association and dissociation rate constants assumed for this simulation were $k_a = 0.69 \times 10^6 M^{-1} min^{-1}$ and $k_d = 3.4 \times 10^{-4} min^{-1}$ (see chapter VI). Left inset: Relative deviation from equilibrium (ε) of specific binding (B_{sp}) as a function of total [^{3}H]dexamethasone concentration (abscissa), with incubation time as the indicated parameter. The rate constants and receptor concentration listed above were input to equation (2-28) to generate the relative error (ε) curves; these curves have maxima in the low nanomolar range and actually decrease to approach constant values as the steroid concentration is reduced indefinitely. Right inset: The effect of inadequate incubation times on the slopes of Scatchard plots derived from "equilibrium" binding isotherms. The binding parameters (rate constants and receptor concentration) listed above were input to equation (2-13) to generate the values of B_{sp} for each indicated incubation time at the seven different steroid concentrations (.5-50 nM). The lines were fit to the resulting points by simple linear regression. The curvature in the simulated data is concentrated in the region near the x-intercept and is not readily apparent upon visual inspection; it is obvious that the apparent linearity of a Scatchard plot is not an adequate criterion for the attainment of equilibrium.


Bsp/Beq
1.0
0.8
0.6
0.4
0.2
50nM
25 nM
10nM
5nM
1 nM
4
8
12
16
20
24
TIME (HRS)
Bsp/F
6
4
2
Equilibrium
24 HR
16HR
12HR
1
2
3
Bsp (nM)
ε = 1 - Bsp / Beq
0.20
0.15
0.10
0.05
12HR
16HR
24HR
10
9
8
-Log Total [3 H] DEX

simulated effect of inadequate incubation time on the slope of the Scatchard plot derived from a "non-equilibrium" binding isotherm. The same binding parameters (rate constants and receptor concentration, listed above) were input to equation (2-13) to generate the values of B_{sp} for each indicated incubation time at the seven different [^{3}H]dexamethasone concentrations (0.5-50 nM) used in the simulated "typical" experiment. The lines were fit to the resulting points in Scatchard space by least-squares linear regression. The curvature in the simulated "nonequilibrium" Scatchard plots is concentrated near the x-intercept; it is not immediately apparent upon visual inspection and does not lead to greatly inflated Hill coefficients. It is thus obvious that this superficial, apparent linearity, which could be masked easily by experimental variance, is not an adequate criterion for the attainment of equilibrium.

The above simulation suggested that our "typical" experiment with [^{3}H]dexamethasone might require a rather lengthy incubation (perhaps 30-36 h), if the affinity was actually as high as the measured rate constants indicated. For example, figure 4-9 (right inset) predicts that a 16 h experiment analyzed by the routine linear Scatchard method might overestimate the true K_d by a factor of approximately 2. Thus, in several experiments we measured specific [^{3}H]dexamethasone binding (B_{sp}) as a function of the time of incubation of cytosol (4 mg/ml) with 1 nM [^{3}H]dexamethasone (the DEAE filter assay was as described above; data not shown). Binding assays were performed after 12, 16, 20, 24 and 40 h incubation with steroid at 2°C; no increase in B_{sp} (which reached approximately 0.6 nM) was found beyond 12 h of incubation, and the binding of [^{3}H]dexamethasone remained unchanged at 40 h. Furthermore,

several isotherms derived from experiments incubated beyond 24 h (up to 36 h) did not yield higher estimates of [^{3}H]dexamethasone affinity than experiments whose incubations were terminated after 16 h. Consequently, equilibrium incubations of cytosol with [^{3}H]dexamethasone were terminated after 16-24 h, in order to minimize the possible slow decapacitation of steroid binding sites that might occur in vitro during very prolonged experiments. A similar prediction based on the measured association and dissociation rate constants for [^{3}H]TA (described in chapter VI) indicated that considerably longer incubations (50-100 h) might be required for the attainment of equilibrium at the lower [^{3}H]TA concentrations used in a typical isotherm experiment (see also Gray and Luttge, 1982); a duration of 48 h was chosen for the [^{3}H]TA experimental incubations, and specific binding was not observed to increase beyond this time. The choice of incubation time for [^{3}H]corticosterone isotherms was not a problem; both simulation (based again on measured rate constants) and observations indicated that specific binding attained a steady state well before the 16-24 h incubations were terminated.

The results of representative individual equilibrium isotherm experiments conducted with the three [^{3}H]glucocorticoid ligands, and some results from merged analyses in which several isotherms were combined, are presented in figures 4-10 through 4-25. Summary results with standard errors for equilibrium binding parameters measured with the three [^{3}H]ligands are presented in table 4-1. Figure 4-10 (derived from the specific binding data of figure 4-7) and figure 4-11 are conventional Scatchard plots depicting the specific binding of

Table 4-1. Equilibrium binding parameters for tritiated glucocorticoids in perfused mouse brain cytosol. The same experimental data (with the indicated exception) have been analyzed by the different methods discussed in the text and in chapter III. The tabulated parameter estimates are either $\bar{x} \pm$ s.e.m. or individual determinations. Numbers within parentheses indicate either units of measure or the number of independent experiments that are averaged or merged. Steroid trivial name abbreviations are defined in the footnotes to table 4-2.

Parameter	Method[a]	[^{3}H]DEX + DEX(200X)	[^{3}H]TA + TA(200X)	[^{3}H]B + DEX(200X)	[^{3}H]B + B(200X)
K_d		($\times 10^{-9}$M)	($\times 10^{-10}$M)	($\times 10^{-9}$M)	($\times 10^{-9}$M)
	A	1.79 ± 0.24 (18)	2.80 ± 0.43 (3)	7.55 ± 0.58 (3)	6.81 ± 0.60 (7)
	B	1.69 ± 0.08 (3)	----	----	----
	C	1.79 ± 0.09[b] (18)	----	----	6.65 ± 0.27[b] (7)
	D	1.90 ± 0.09[b] (18)	----	----	7.06 ± 0.26[b] (7)
	E	1.54 ± 0.11[b] (18)	----	----	7.04 ± 0.04[b] (7)
	F	1.72[c] (18)	----	----	6.18[c] (7)

Continued

Table 4-1. (Continued)

Parameter	Method[a]	[^{3}H]DEX ± DEX(200X)	[^{3}H]TA ± TA(200X)	[^{3}H]B ± DEX(200X)	[^{3}H]B ± B(200X)
K_d		($\times 10^{-9}$M)	($\times 10^{-10}$M)	($\times 10^{-9}$M)	($\times 10^{-9}$M)
	G	1.74 (18)	----	----	4.57 (7)
	H	0.46 ± 0.07 (3)	0.34 (1)	9.52 ± 1.06 (3)	----
Hill "n"					
	I	1.01 ± 0.03 (15)	1.04 ± 0.08 (3)	1.01 ± 0.02 (3)	0.95 ± 0.03 (7)
	F	0.99[d] (18)	----	----	0.95[d] (7)
B_0 or B_{MAX}		(fmole/mg protein)	(fmole/mg protein)	(fmole/mg protein)	(fmole/mg protein)
	A	374 ± 15 (43)[e]	397 ± 32 (3)	364 ± 59 (3)	602 ± 36 (15)[f]

a. Methods used for the analysis of binding data are A: individual Scatchard plots (B_{sp} input, simple linear regression); B: individual Scatchard plots (constant ligand, variable cytosol concentration, B_{sp} input, simple linear regression); C: merged analysis (B_T and B_0 are input, weighted nonlinear

Continued

Table 4-1. (Continued)

regression with one specific and one nonspecific component); D: merged analysis (similar to C, but B_{sp} is input to find one specific component only); E: merged free receptor plot (2-parameter simple linear regression performed with nonlinear regression program); F: merged Hill plot (20%-80% occupancy only, fit by IMSL cubic spline subroutine); G: merged affinity spectrum (a transform of the log (B_T/B_0) vs. -log F binding plot smoothed by cubic spline); H: ratio of measured rate constants (described in chapter VI); I: individual Hill plots (occupancy 0%-80%, simple linear regression).

b. "Asymptotic" s.e.m. generated by SAS nonlinear regression program (Ralston and Jennrich, 1978).

c. Determined from abscissa at 50% occupancy (i.e., logit $[B_{sp}/B_0]=0$).

d. Slope at 50% occupancy.

e. Includes 25 single ligand concentration ("saturation") assays.

f. Includes 9 single ligand concentration determinations.

Fig. 4-10. Scatchard plot (derived from the specific binding data of figure 4-7) depicting the binding of [^{3}H]dexamethasone to glucocorticoid receptors in perfused mouse brain cytosol.

Experimental conditions are described in the legend to figure 4-7 and in Methods. The line was fit by least-squares linear regression. Cytosol protein concentration was 6.6 mg/ml. Equilibrium dissociation constant K_d = 1.2 nM, and receptor concentration B_0 = 2.5 nM (380 fmole/mg cytosol protein). Inset: Hill plot (line fit by simple linear regression) derived from the same specific binding data combined with the estimate of B_0 generated by the Scatchard plot. Regression includes points in the region where receptor occupancy ($\theta = B_{sp}/B_0$) does not exceed .8 (or 80%). The slope of the line provides the estimated Hill coefficient n = 1.03.

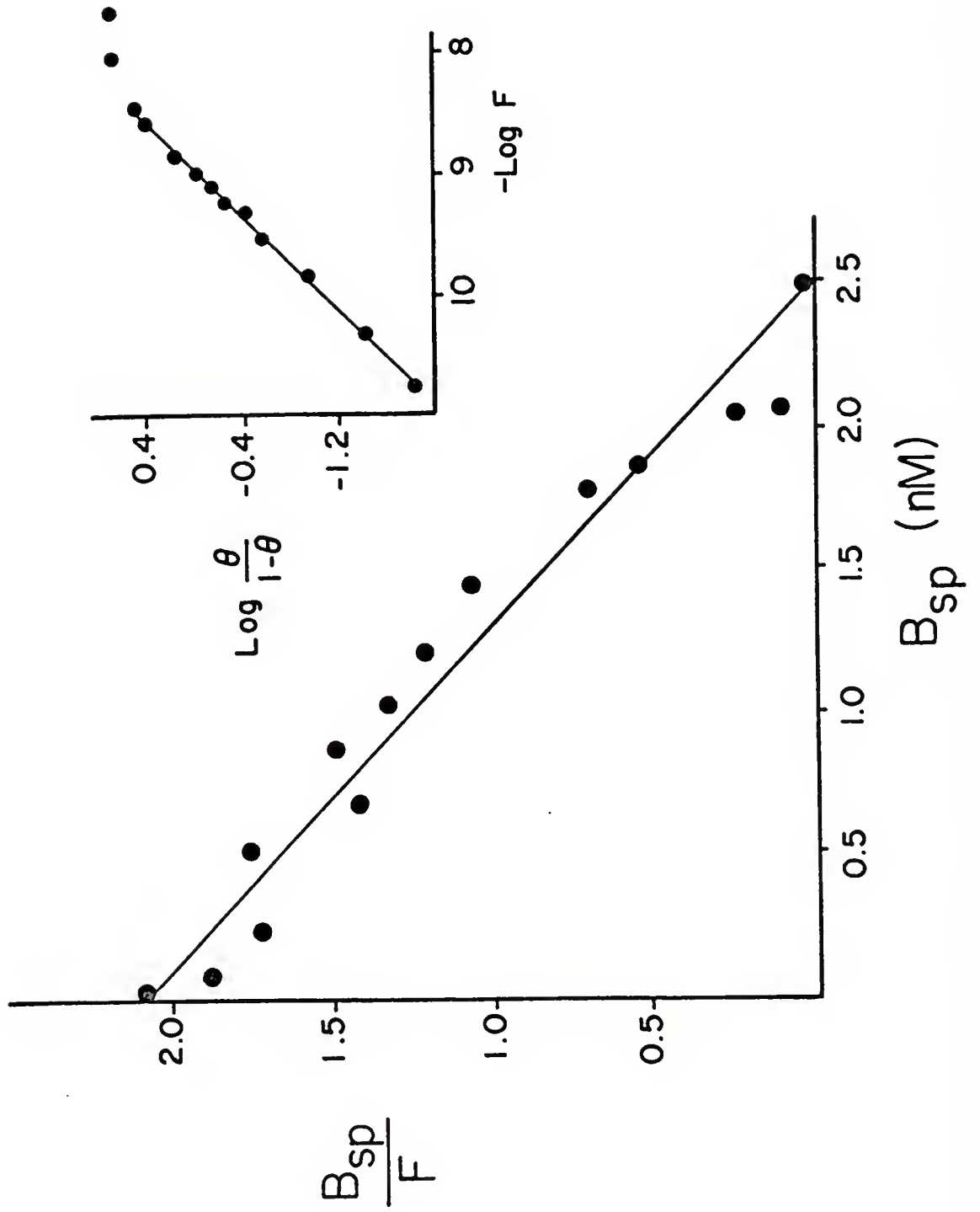

$\frac{B_{sp}}{F}$
2.0
1.5
1.0
0.5
0.5
1.0
1.5
2.0
2.5
B_{sp} (nM)
$\text{Log } \frac{\theta}{1-\theta}$
0.4
-0.4
-1.2
10
9
8
-Log F

Fig. 4-11. Representative Scatchard plot depicting the binding of [^{3}H]triamcinolone acetonide (TA) to glucocorticoid receptors in perfused mouse brain cytosol.

Cytosol was prepared in buffer A, and pairs of samples (250 µl) were incubated for 48 h at 2°C with increasing concentrations of [^{3}H]TA and, for one of each sample pair, a 200-fold excess of unlabeled TA. 50 µl aliquots were then assayed in triplicate by the DEAE filter method for the determination of specifically bound steroid (B_{sp}). For the specific experiment illustrated above the cytosol protein concentration was 5.5 mg/ml, and the total [^{3}H]TA concentration ranged from 0.32 to 17.6 nM. The line was fit by simple least-squares regression. Equilibrium dissociation constant K_d = 0.36 nM, and receptor concentration B_0 = 2.2 nM (410 fmole/mg cytosol protein). Inset: Hill plot derived from the same specific binding data combined with the estimate of B_0 generated by the Scatchard plot. Hill coefficient n=1.00 (regression restricted to the region of occupancy $\theta = B_{sp}/B_0$ not exceeding .8 or 80%).

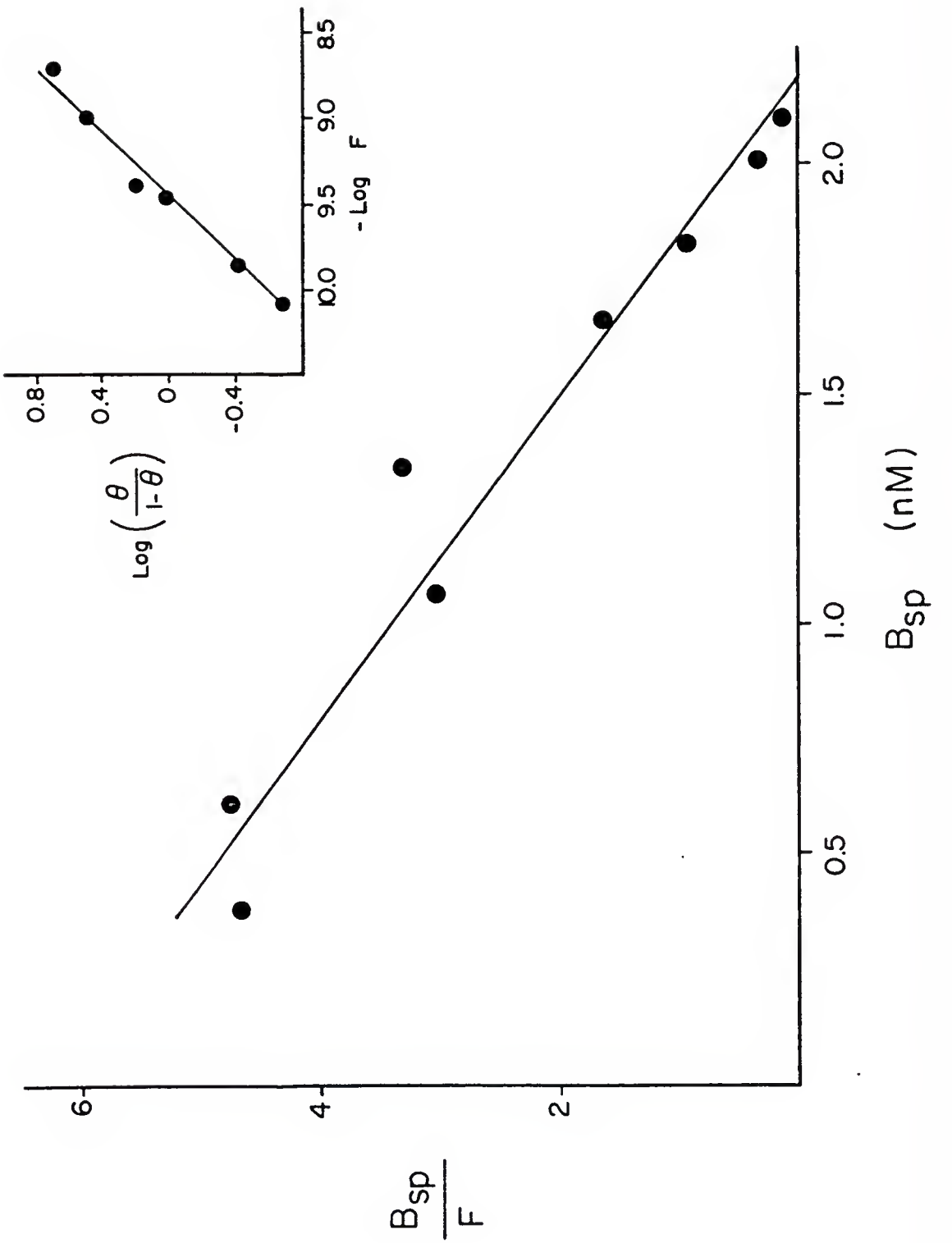

$\frac{B_{sp}}{F}$
6
4
2
0.5
1.0
1.5
2.0
B_{sp} (nM)
$\text{Log}\left(\frac{\theta}{1-\theta}\right)$
0.8
0.4
0
-0.4
10.0
9.5
9.0
8.5
- Log F

Fig. 4-12. Scatchard plot (derived from the specific binding data of figure 4-8) depicting the binding of [^{3}H]corticosterone to the complete ensemble of saturable corticosterone binding sites in perfused mouse brain cytosol.

Experimental conditions are described in the legend to figure 4-8 and in Methods. The line was fit by simple least-squares linear regression. Cytosol protein concentration was 7.0 mg/ml. The data are deceptively consistent with a single, apparently homogeneous class of high-affinity binding sites. Apparent equilibrium dissociation constant K_d = 8.4 nM, and total binding site concentration B_{MAX} = 4.2 nM (600 fmole/mg cytosol protein). Inset: Hill plot derived from the same specific binding data combined with the estimate of B_{MAX} generated by the Scatchard plot. Hill coefficient n = 1.03 (restricted to the region of occupancy $\theta = B_{sp}/B_{MAX}$ not exceeding .8 or 80%).

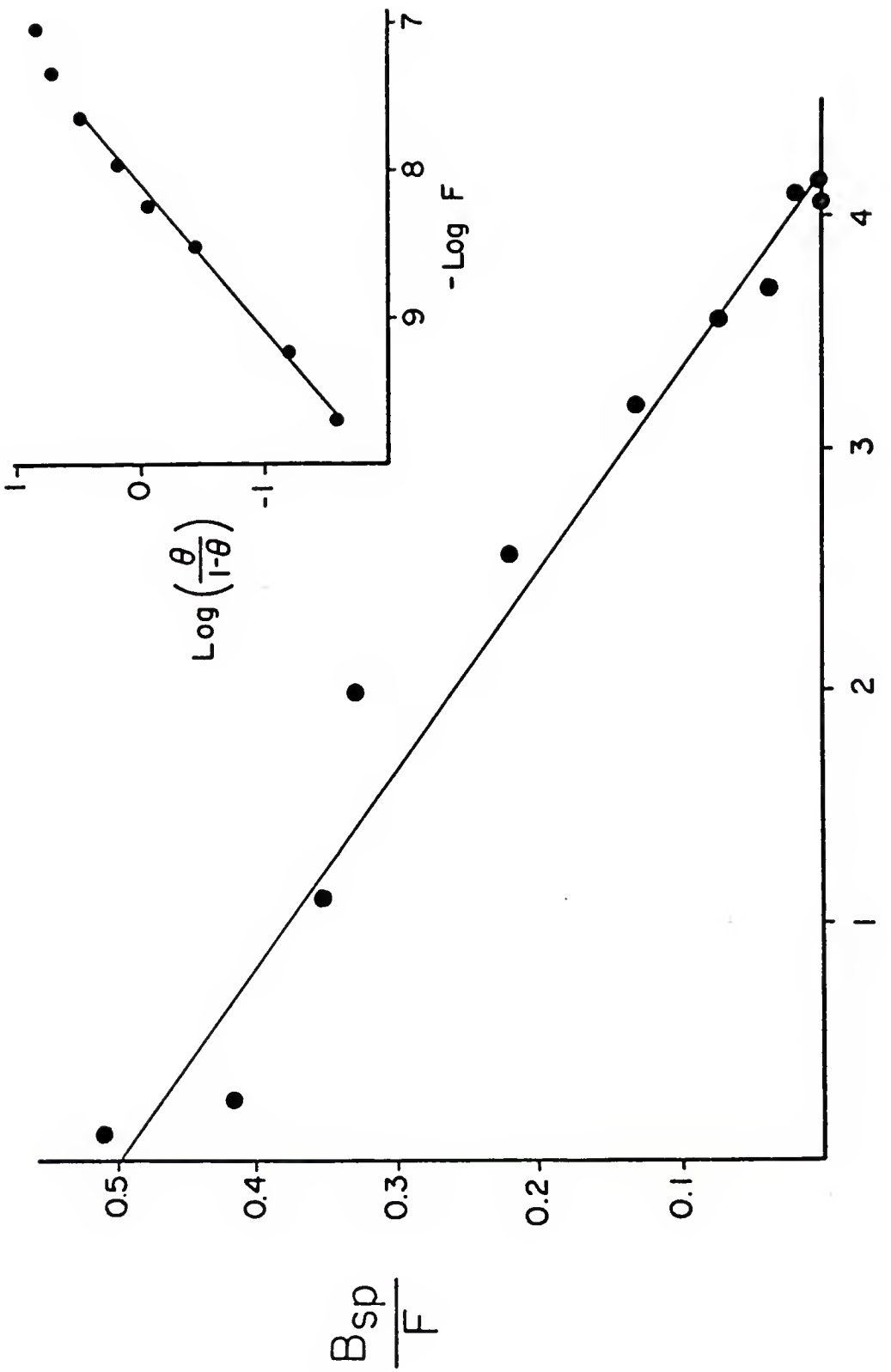

0.5
0.4
0.3
0.2
0.1
$\frac{B_{sp}}{F}$
1
2
3
4
B_{sp} (nM)
1
0
-1
$\text{Log}\left(\frac{\theta}{1-\theta}\right)$
7
8
9
-Log F

Fig. 4-13. Representative Scatchard plot depicting the binding of [^{3}H]corticosterione to glucocorticoid receptors in perfused mouse brain cytosol.

Cytosol was prepared in buffer A, and pairs of samples (250 µl) were incubated for 16 h at 2°C with increasing concentrations of [^{3}H]corticosterone and, for one of each sample pair, a 200-fold excess of unlabeled dexamethasone. 50 µl aliquots were assayed in triplicate by the DEAE filter method for the determination of specifically bound [^{3}H]corticosterone that can be displaced by dexamethasone. The cytosol protein concentration was 3.8 mg/ml, and total [^{3}H]corticosterone concentration range was 2.9-170 nM. The line was fit by simple least-squares regression. Equilibrium dissociation constant K_d = 8.6 nM, and receptor concentration B_0 = 1.8 nM (468 fmole/mg cytosol protein). Inset: Hill plot derived from the same specific binding data combined with the estimate of B_0 generated by the Scatchard plot. Hill coefficient n = 0.97 (occupancy $\theta = B_{sp}/B_0$ not exceeding .8 or 80%).

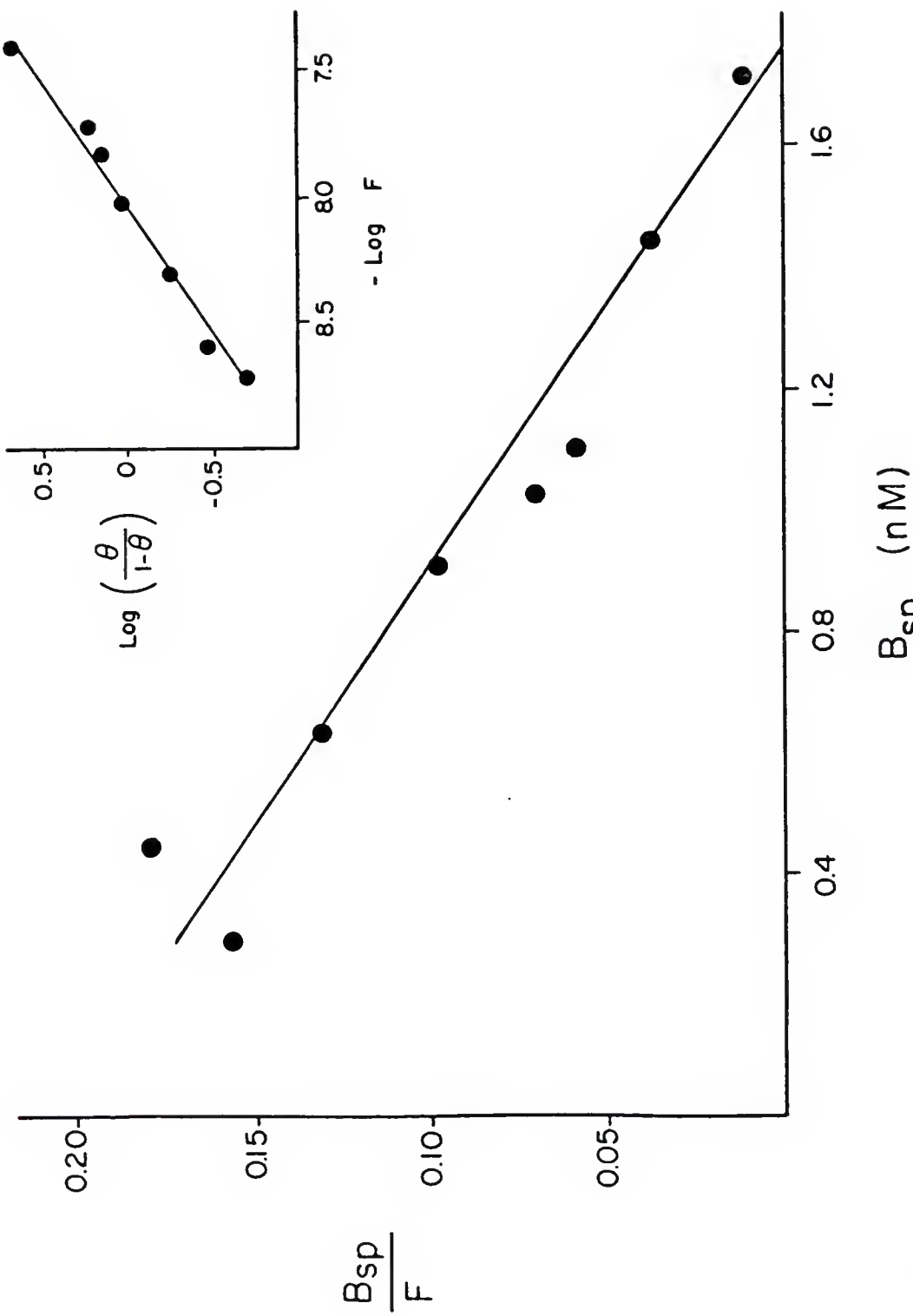

0.20
0.15
0.10
0.05
Bsp/F
0.4
0.8
1.2
1.6
Bsp (nM)
0.5
0
-0.5
Log (θ/1-θ)
7.5
8.0
8.5
- Log F

Fig. 4-14. Nonlinear regression analysis of the binding of [^{3}H]dexamethasone to glucocorticoid receptors in perfused mouse brain cytosol (derived from the total binding data, B_T, of figure 4-7 and displayed in the Scatchard coordinate system).

Experimental conditions are indicated in the legend to figure 4-7, and the use of the nonlinear regression program is described in Methods. The model to which the data were fit consists of the sum of one saturable high-affinity (specific) component and one nonsaturable (nonspecific) component as described by equation (4-6). The actual regression equation does not contain F; it accepts B_T and S_L (total [^{3}H]dexamethasone concentration) as pairs of input variables and then regresses B_T on the relatively error-free variable S_L, fitting the adjustable binding parameters K_d, B_0, and C_1 (the asymptotic "sink" of nonspecific binding). Each input data point was weighted as $1/B_T^2$, consistent with the assumption that the coefficient of variation or percentage error in the measurement of B_T is constant over the range of B_T. For display the resulting complete 3-parameter regression model was then plotted in the Scatchard coordinate system (curved line), and each component was also plotted separately (2 straight lines). (Note that the x-axis is plotted below y=0 in the figure.) Equilibrium dissociation constant K_d = 1.1 nM, and receptor concentration B_0 = 2.4 nM (370 fmole/mg cytosol protein). $C_1 = 2.4 \times 10^6\ M^{-1}$. Agreement with the binding parameter estimates derived from the Scatchard plot of specific binding fit by simple linear regression (figure 4-10) is excellent.

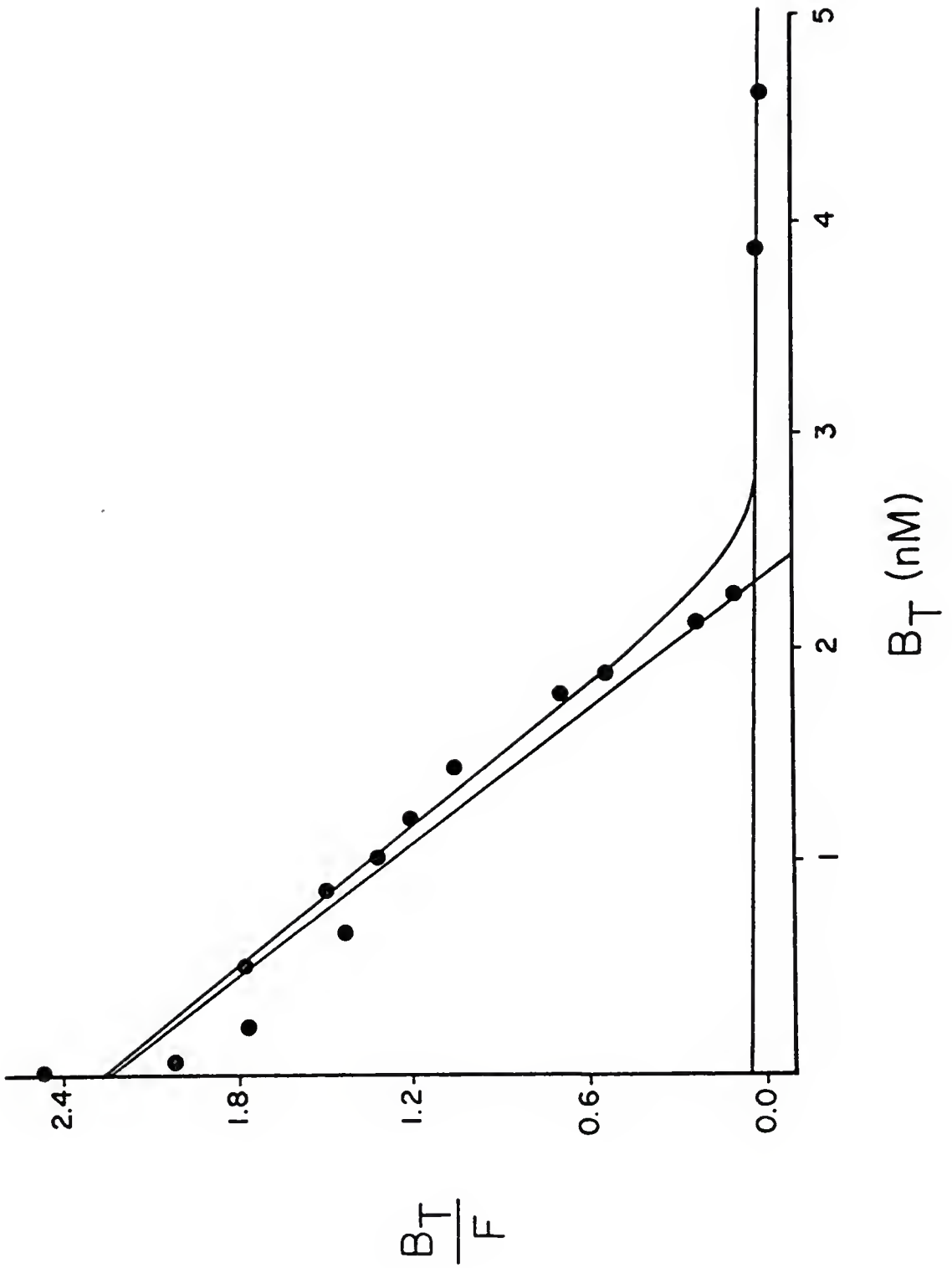

2.4
1.8
1.2
0.6
0.0
$\frac{B_T}{F}$
1
2
3
4
5
B_T (nM)

Fig. 4-15. Nonlinear regression analysis of the binding of [^{3}H]corticosterone to the complete ensemble of saturable corticosterone binding sites in perfused mouse brain cytosol (derived from the total binding data, B_T, of figure 4-8 and displayed in the Scatchard coordinate system).

Experimental conditions are indicated in the legend to figure 4-8, and the use of the nonlinear regression program is described in Methods. The model to which the data were fit consists of the sum of one saturable high-affinity (specific) component and one nonsaturable (nonspecific) component as described by equation (4-6). The actual regression equation does not contain F; it accepts B_T and S_L (total [^{3}H]corticosterone concentration) as pairs of input variables and then regresses B_T on the relatively error-free variable S_L, fitting the adjustable binding parameters K_d, B_{MAX} (which replaces B_0 in equation 4-6), and C_1 (the asymptotic "sink" of nonspecific binding). Each input data point was weighted as $1/B_T^2$, consistent with the assumption that the coefficient of variation or percentage error in the measurement of B_T is constant over the range of B_T. For display the resulting complete 3-parameter regression model was plotted in the Scatchard coordinate system (curved line), and each component was also plotted separately (2 straight lines). (The x-axis is plotted below y=0 in the figure.) Apparent equilibrium dissociation constant K_d = 8.8 nM, and total binding site concentration B_{MAX} = 4.6 nM (656 fmole/mg cytosol protein.) $C_1 = 0.73 \times 10^6\ M^{-1}$. Agreement with the binding parameter estimates derived from the Scatchard plot of specific binding fit by simple linear regression (figure 4-12) is excellent.

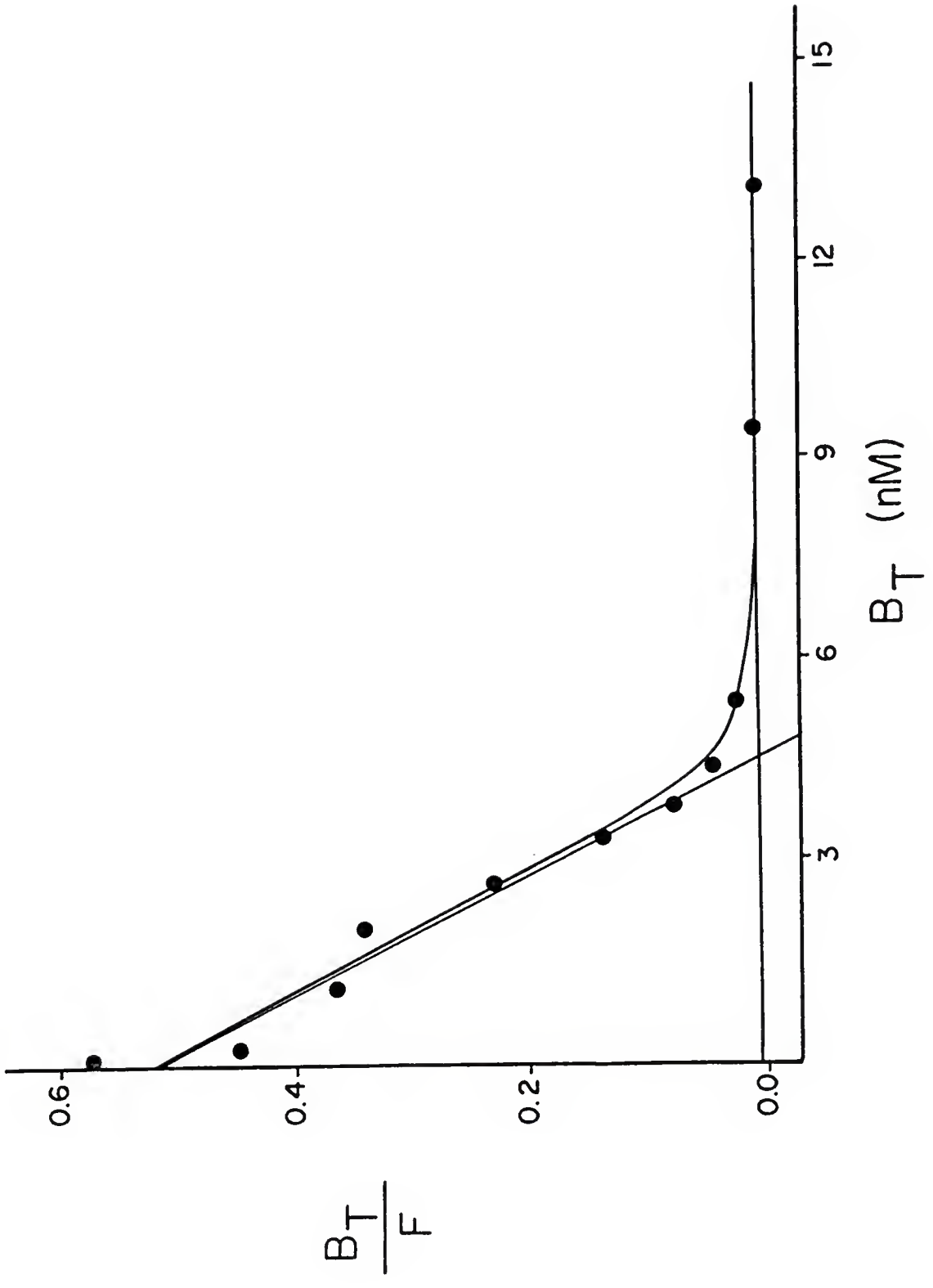

0.6
0.4
0.2
0.0
B_T / F
3
6
9
12
15
B_T (nM)

Fig. 4-16. Merged nonlinear regression analysis of the binding of [^{3}H]dexamethasone to glucocorticoid receptors in the perfused mouse brain cytosol (derived from the values of total binding, B_T, from 18 independent experiments) combined with the individual estimates for each experiment, of the receptor concentration (B_0) derived from initial Scatchard plots such as that depicted in figure 4-10).

The data and the resulting regression have been plotted in a "Scatchard-like" coordinate system (where B_{sp} or B_T has been replaced by B_T/B_0, the normalized value of total bound steroid). Representative experimental conditions are indicated in the legend to figure 4-7, and the use of the nonlinear regression program is described in Methods. The model to which the data were fit consists of the sum of one saturable high-affinity (specific) component and one nonsaturable (nonspecific) component as described by equation (4-6). The actual merged regression equation does not contain F; it accepts B_T, B_0, and S_L (total [^{3}H]dexamethasone concentration) as sets of input variables and then regresses B_T on the variables S_L and B_0, fitting the adjustable binding parameters K_d and C_1 (the asymptotic "sink" of nonspecific binding). Each input data point was weighted as $1/B_T^2$, consistent with the assumption that the coefficient of variation or percentage error in the measurement of B_T is constant over the range of B_T. For display the resulting complete 2-parameter regression model was plotted in the "Scatchard-like" coordinate system (curved line), and each component was also plotted separately (2 straight lines). (Note that the x-axis is plotted below y=0 in the figure.) Equilibrium dissociation constant K_d = 1.8 ± 0.1 nM (n = 18 experiments, 127 points). $C_1 = 2.5 \times 10^6\ M^{-1}$. Range of total receptor concentration B_0: 2.1 - 3.9 nM; range of cytosol protein concentration 5.3 - 13.3 mg/ml.

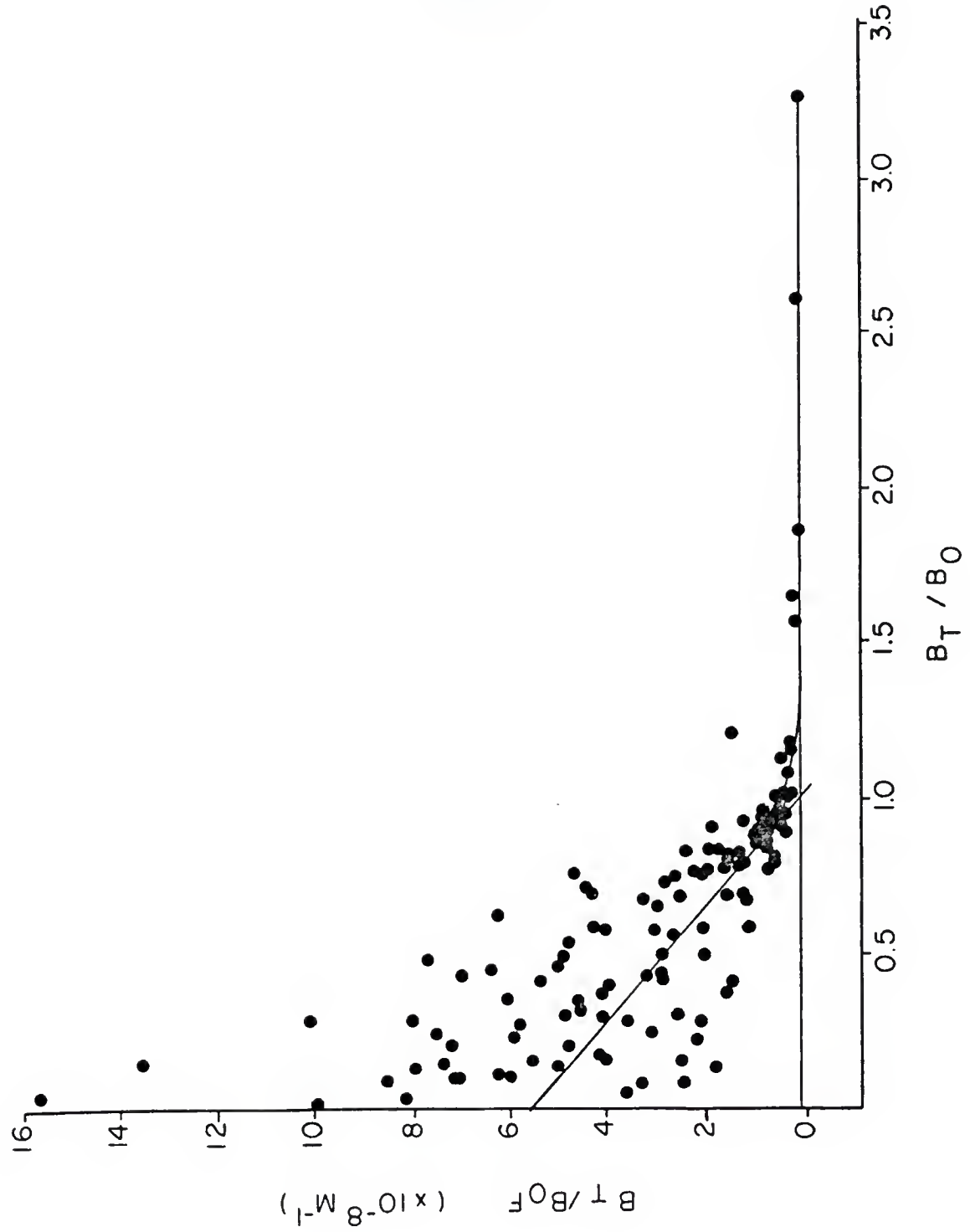

B_T / B_O
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
B_T/B_OF ($\times 10^{-8}$ M^{-1})
0
2
4
6
8
10
12
14
16

Fig. 4-17. Merged nonlinear regression analysis of the binding of [^{3}H]corticosterone to the complete ensemble of corticosterone binding sites in perfused mouse brain cytosol (derived from the values of total binding, B_T, from 7 independent experiments combined with the individual estimates, for each experiment, of the receptor concentration, B_{MAX}, derived from initial Scatchard plots such as that depicted in figure 4-12).

The data and the resulting regression have been plotted in a "Scatchard-like" coordinate system (where B_{sp} or B_T has been replaced by B_T/B_{MAX}, the normalized value of total bound steroid). Representative experimental conditions are indicated in the legend to figure 4-8, and the use of the nonlinear regression program is described in Methods. The model to which the data were fit consists of the sum of one saturable high-affinity (specific) component and one nonsaturable (nonspecific) component as described by equation (4-6). The merged regression equation does not contain F; it accepts B_T, B_{MAX} (substituted for B_0), and S_L (total [^{3}H]corticosterone concentration) as sets of input variables and then regresses B_T on the variables S_L and B_{MAX}, fitting the adjustable binding parameters K_d and C_1 (the asymptotic "sink" of nonspecific binding). Each input data point was weighted as $1/B_T^2$, consistent with the assumption that the coefficient of variation or percentage error in the measurement of B_T is constant over the range of B_T. For display the resulting complete 2-parameter regression model was plotted in the "Scatchard-like" coordinate system (curved line), and each component was also plotted separately (2 straight lines). (Note that the x-axis is plotted below y=0 in the figure.) Equilibrium dissociation constant K_d = 6.6 ± 0.3 nM (n = 7 experiments, 50 points). $C_1 = 0.88 \times 10^6\ M^{-1}$. Range of total binding site concentration B_{MAX}: 4.1 - 7.5 nM; range of cytosol protein concentration 4.8 - 9.3 mg/ml.

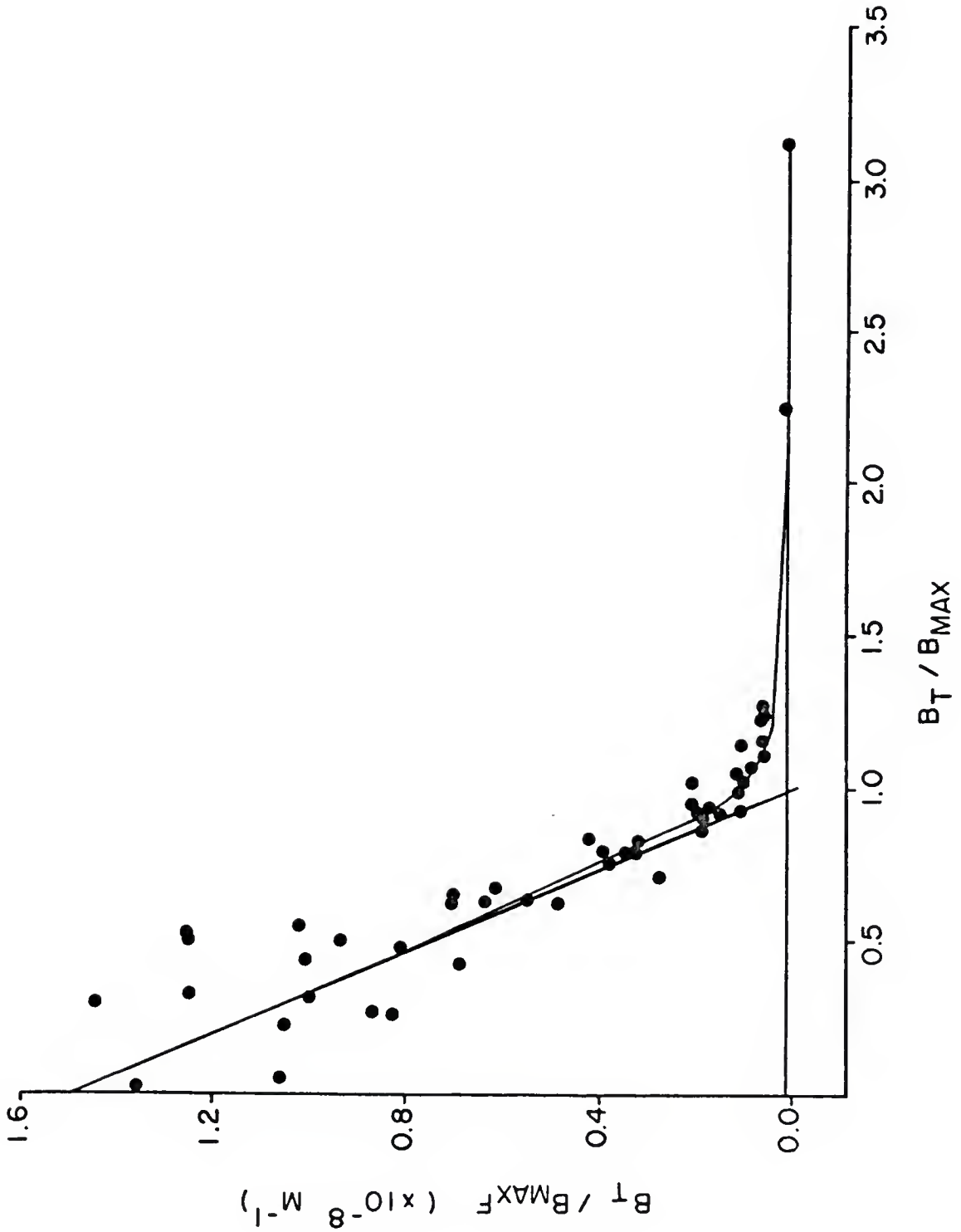

1.6
1.2
0.8
0.4
0.0
B_T / B_{MAXF} ($\times 10^{-8}$ M^{-1})
0.5
1.0
1.5
2.0
2.5
3.0
3.5
B_T / B_{MAX}

Fig. 4-18. Merged Hill plot analysis of the binding of [^{3}H]dexamethasone to glucocorticoid receptors in the perfused mouse brain cytosol (derived from 62 values of specific binding, B_{sp}, from 18 independent experiments combined with the individual estimtes, for each experiment, of the receptor concentration (B_o) derived from initial Scatchard plots such as that depicted in figure 4-10).

Only data points corresponding to a receptor occupancy ($\theta = B_{sp}/B_o$) between .2 (20%) and .8 (80%) were included in the analysis. The curve (almost linear) was fit by a cubic spline algorithm that employs polynomials to smooth the data in accordance with a statistical criterion; this method of regression contains no assumptions (other than the smoothness criterion) concerning the basic form or shape of the curve, and thus is appropriate for empirically determining both the shape of the Hill plot and the abscissa and slope at the 50% occupancy point (where logit $\theta = 0$). Representavie experimental conditions are indicated in the legend to figure 4-7, and the use of the IMSL library cubic spline subroutine is described in Methods. Equilibrium dissociation constant K_d = 1.7 nM (abscissa at logit $\theta = 0$); Hill coefficient (slope at logit $\theta = 0$) n = 0.99. Range of total receptor concentration B_o:2.1 - 3.9 nM; range of cytosol protein concentration 5.3 - 13.3 mg/ml.

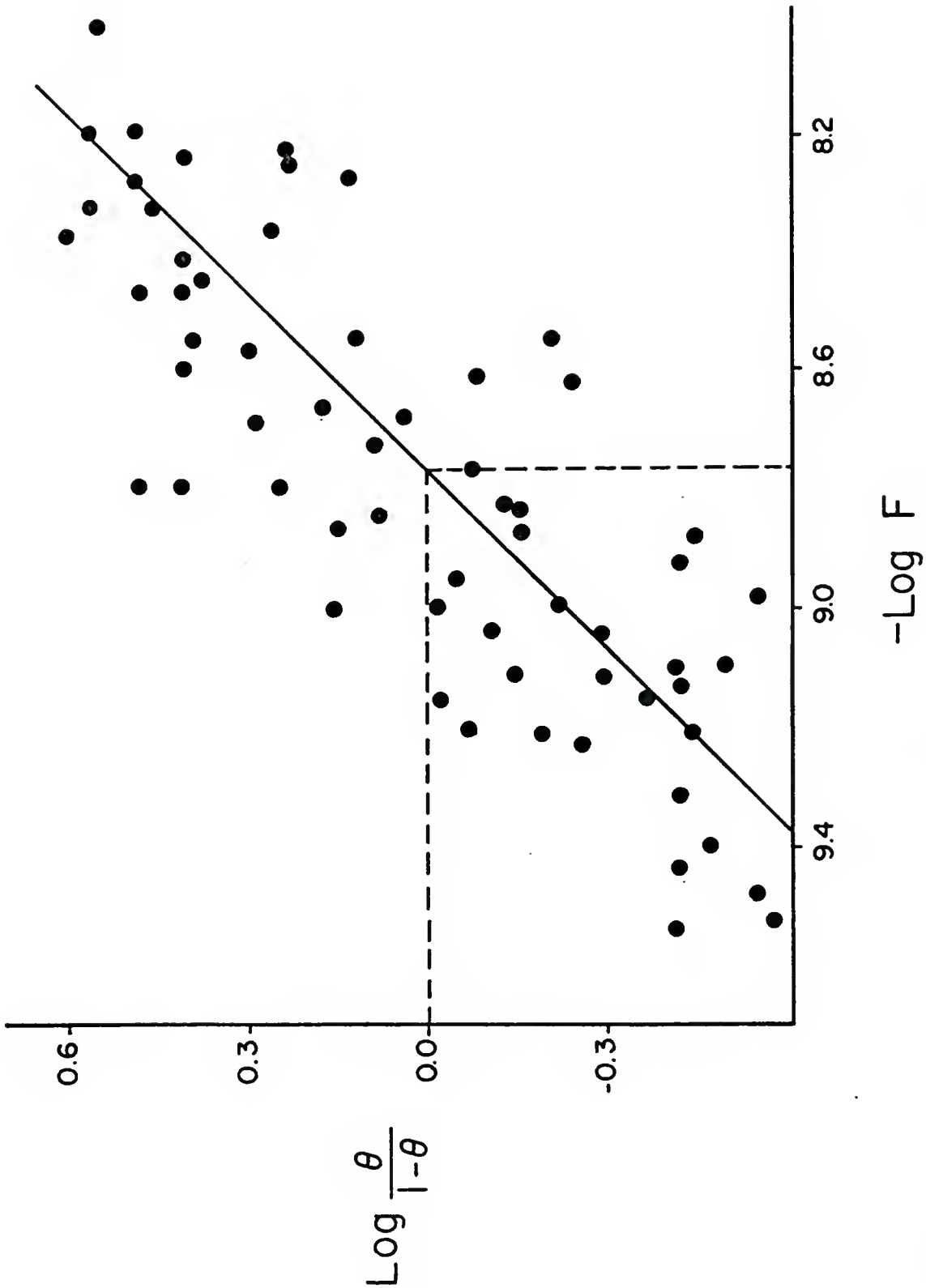

0.6
0.3
0.0
-0.3
Log θ/(1-θ)
8.2
8.6
9.0
9.4
-Log F

Fig. 4-19. Merged Hill plot of the binding of [^{3}H]corticosterone to the complete ensemble of corticosterone binding sites in perfused mouse brain cytosol (derived from 28 values of specific binding, B_{sp}, from 7 independent experiments combined with the individual estimates, for each experiment, of the total binding site concentration, B_{MAX}, derived from initial Scatchard plots such as that depicted in figure 4-12).

Only points corresponding to binding site occupancy ($\theta = B_{sp}/B_{MAX}$) between .2 (20%) and .8 (80%) were included in the analysis. The curve (almost linear) was fit by a cubic spline algorithm that employs polynomials to smooth data in accordance with a statistical criterion; this method of regression contains no assumptions (other than the smoothness criterion) concerning the basic form or shape of the curve, and thus is appropriate for empirically determining the shape of the Hill plot and the abscissa and slope at 50% occupancy (logit $\theta = 0$). Representative experimental conditions are indicated in the legend to figure 4-8, and the use of the IMSL library cubic spline subroutine is described in Methods. Equilibrium dissociation constant K_d = 6.2 nM (abscissa at logit $\theta = 0$); Hill coefficient (slope at logit $\theta = 0$) n = 0.95. Range of total binding site concentration B_{MAX}: 4.1 - 7.5 nM; range of cytosol protein concentration 4.8 - 9.3 mg/ml.

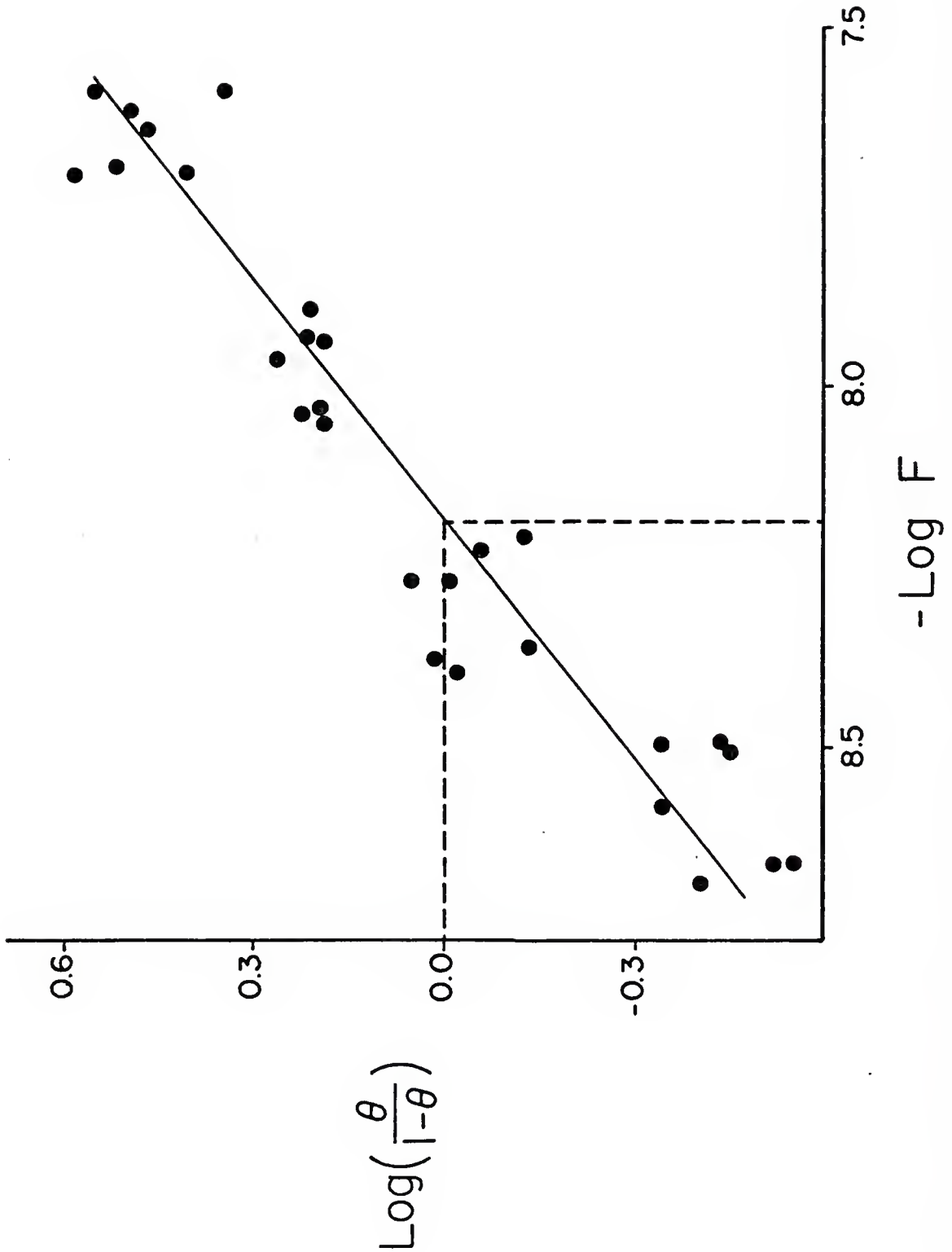

0.6
0.3
0.0
-0.3
7.5
8.0
8.5
-Log F
Log(θ/1-θ)

Fig. 4-20. Merged "free receptor" plot of the binding of [^{3}H]dexamethasone to glucocorticoid receptors in perfused mouse brain cytosol (derived from the values of specific binding, B_{sp}, from 18 independent experiments combined with the individual estimates, for each experiment, of the receptor concentration B_0 derived from initial Scatchard plots such as that depicted in figure 4-10).

Representative experimental conditions are indicated in the legend to figure 4-7, and the use of the nonlinear regression program to generate a standard error for the slope of the line is described in Methods. The line was fit by unweighted, 2-parameter least-squares linear regression and thus was not constrained by the regression equation to pass through the origin. The ordinate is that of the normal Scatchard plot, but the abscissa has been changed to the free receptor concentration (B_0-B_{sp}) to allow the merging of experiments having different total receptor concentrations. Equilibrium dissociation constant (reciprocal of the slope) K_d = 1.5 ± 0.1 nM (n=18 experiments, 127 points). Range of total receptor concentration B_0: 2.1 - 3.9 nM; range of cytosol protein concentration 5.3 - 13.3 mg/ml.

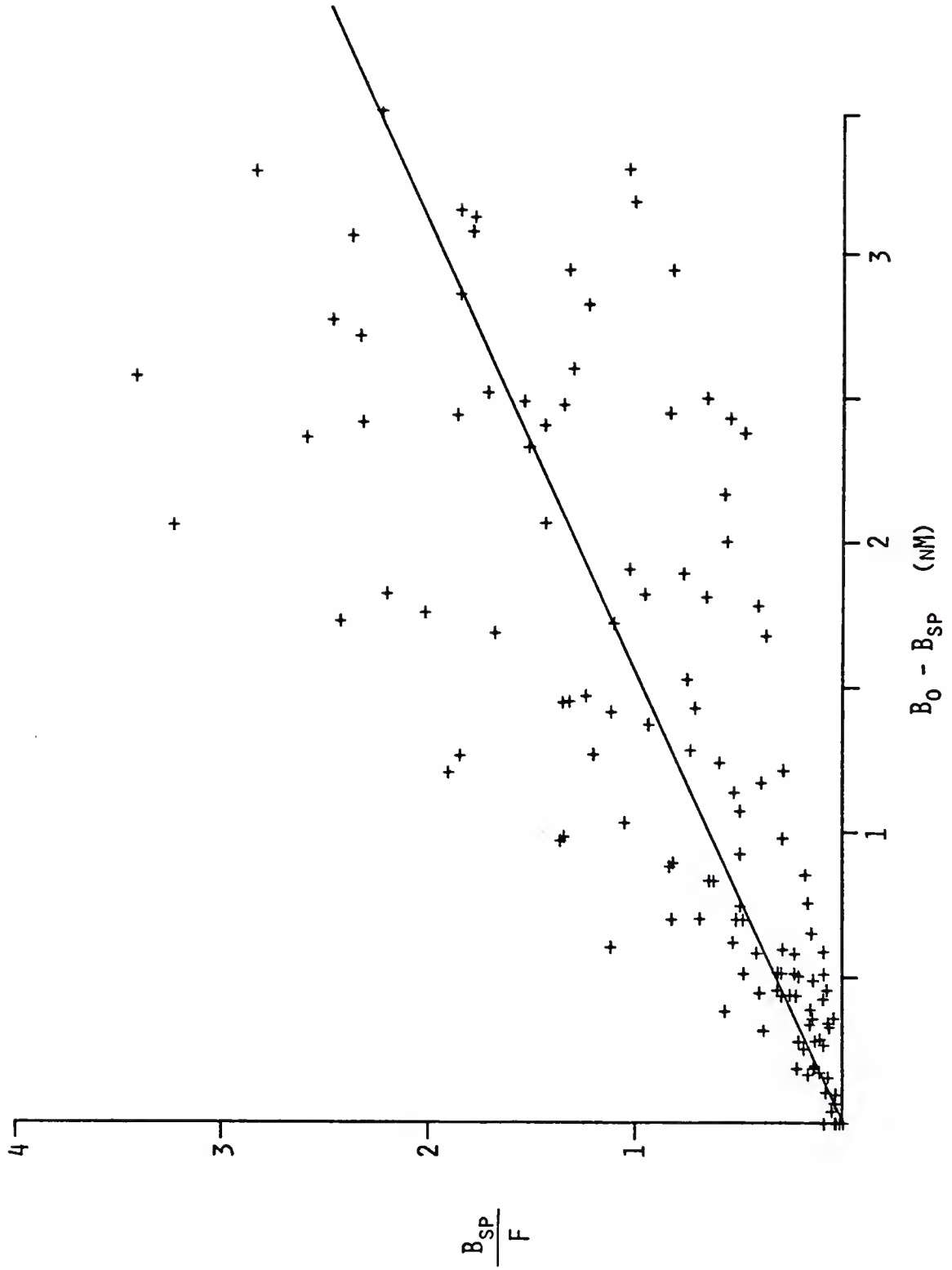

4
3
2
1
$\frac{B_{SP}}{F}$
1
2
3
$B_0 - B_{SP}$ (NM)

Fig. 4-21. Merged "free receptor" plot of the binding of [^{3}H]corticosterone to the complete ensemble of corticosterone binding sites in perfused mouse brain cytosol (derived from the values of specific binding, B_{sp}, from 7 independent experiments combined with the individual estimates, for each experiment, of the total binding site concentration B_{MAX}, derived from initial Scatchard plots such as that depicted in figure 4-12).

Representative experimental conditions are indicated in the legend to figure 4-8, and the use of the nonlinear regression program to generate a standard error for the slope of the line is described in Methods. The line was fit by unweighted, least-squares linear regression and was not constrained by the regression model to pass through the origin. The ordinate is that of the normal Scatchard plot, but the abscissa has been changed to the free receptor concentration (B_{MAX}-B_{sp}) to allow the merging of experiments having different total binding site concentrations and to display the resulting pattern of variance. Equilibrium dissociation constant (reciprocal of the slope) K_d = 7.0 ± 0.1 nM (n=7 experiments, 50 points). Range of total binding site concentration B_{MAX}: 4.1 - 7.5 nM, range of cytosol protein concentration 4.8 - 9.3 mg/ml.

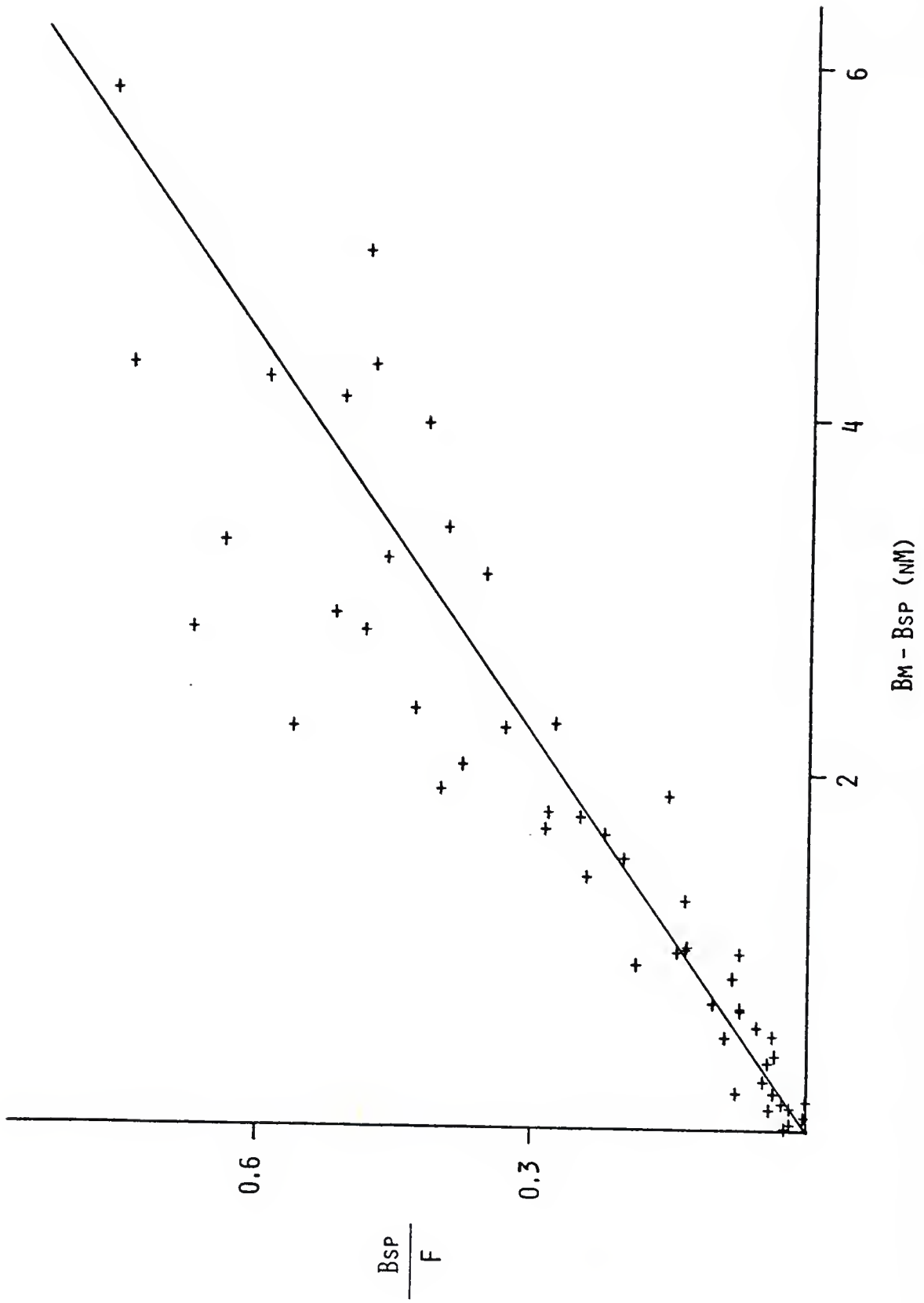

0.6
0.3
Bsp / F
2
4
6
Bm - Bsp (nm)

Fig. 4-22. Affinity spectral analysis of the binding of [^{3}H]dexamethasone to glucocorticoid receptors in perfused mouse brain cytosol (derived from the total binding data, B_T, of the experiment shown in figure 4-7).

Experimental conditions are described in the legend to figure 4-7, and the use of the approximate method of finite differences is described in Methods. Affinity distribution A is a transformation of binding data "predicted" by the complete 3-parameter nonlinear regression displayed as the curved line in figure 4-14 (i.e., binding data that have been "smoothed" by force-fit to a specific binding model); thus spectrum A is the representation of a single homogenous class of high-affinity binding sites. Distribution B is a transformation of total binding data that have been smoothed by the relatively "assumption-free" cubic spline algorithm described in Methods. In both cases the transformation was applied to binding functions expressed in the log B_T-log F coordinate system. To generate spectrum A the curved regression line of figure 4-14 was transformed to the log B_T-log F coordinate system before use of the finite differences method; spectrum B was produced by smoothing the experimental B_T data in this same coordinate system with the cubic spline algorithm before transformation to the affinity distribution. Both the unconstrained spectrum and the comparison spectrum have been normalized by the total receptor concentration (B_0) estimated by the regression displayed in figure 4-14. Spectrum A (equivalent to the regression of figure 4-14): equilibrium dissociation constant K_d = 1.1 nM; spectrum B (data smoothed by "model-free" cubic spline): equilibrium dissociation constant K_d = 1.1 nM. Method of finite differences: input spacing log K = 0.1, log a = 0.2. N(K) is dimensionless; the dimensionality of K is M^{-1} (liters/mole). Inset: Total binding (B_T) data of figure 4-7 smoothed by cubic spline algorithm in the log B_T-log F coordinate system (B_T data points not shown). The subroutine uses a statistical criterion to smooth the data, but there is no force-fit to any specific biophysical model as in the regression of figure 4-14. The "spline-smoothed" binding curve was transformed into spectrum B by the method of finite differences (Thakur et al., 1980).

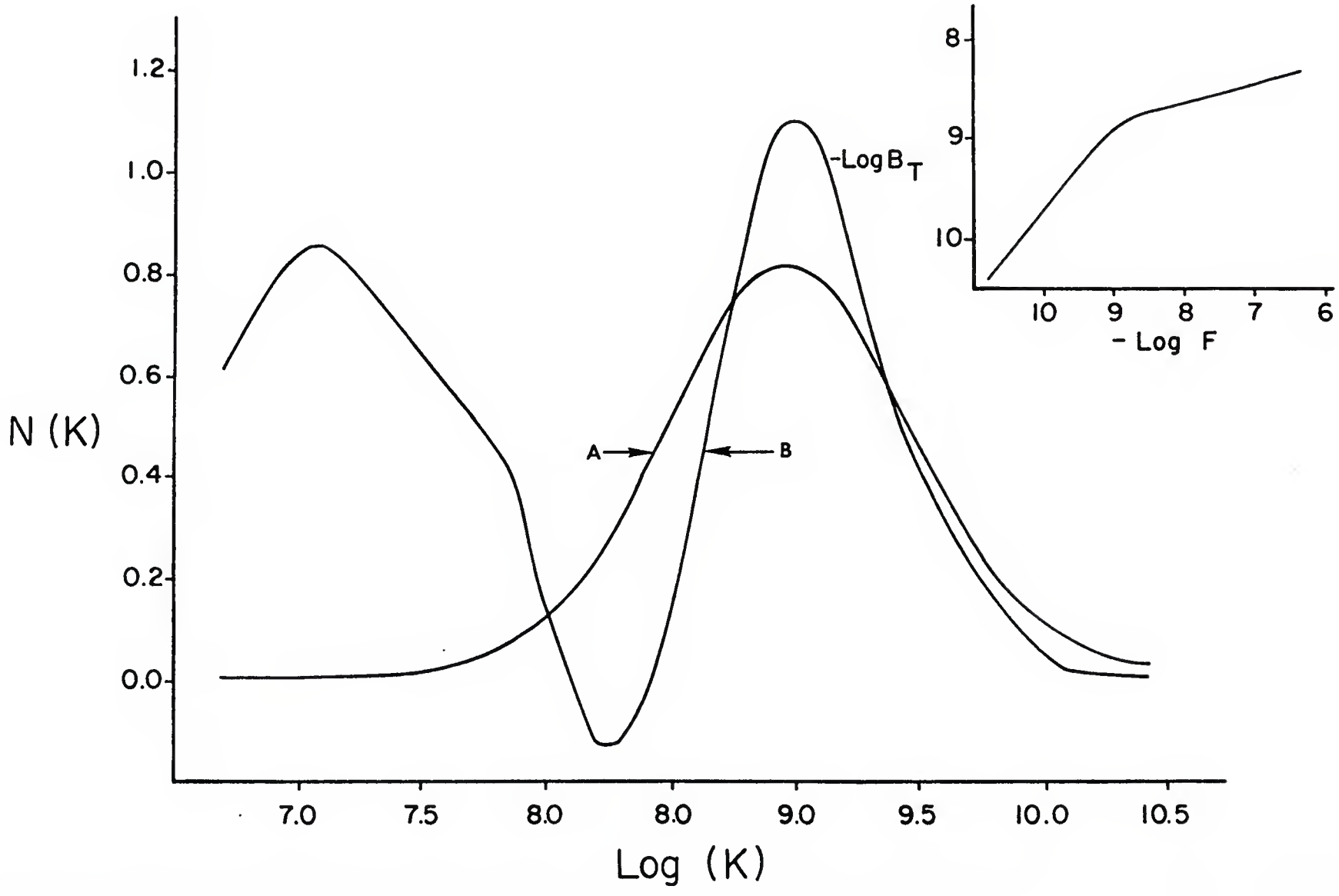

1.2
1.0
0.8
0.6
0.4
0.2
0.0
N (K)
7.0
7.5
8.0
8.0
9.0
9.5
10.0
10.5
Log (K)
A
B
-Log B T
8
9
10
10
9
8
7
6
- Log F

Fig. 4-23. Affinity spectral analysis of the binding of [^{3}H]corticosterone to the complete ensemble of corticosterone binding sites is perfused mouse brain cytosol (derived from the total binding data, B_T, of the experiment shown in figure 4-8).

Experimental conditions are described in the legend to figure 4-8, and the use of the approximate method of finite differences is described in Methods. Distribution A is a transformation of binding data "predicted" by the complete 3-parameter regression displayed as the curve in figure 4-15 (i.e., binding data that have been "smoothed" by force-fit to a specific binding model); thus, spectrum A is the representation of a single homogeneous class of high-affinity binding sites. Distribution B is a transforamtion of total binding data that have been smoothed by the relatively "assumption-free" cubic spline algorithm described in Methods. In both cases the transformation method was applied to binding functions expressed in the log B_T-log F coordinate system. To generate spectrum A the curved regression line of figure 4-15 was transformed to the log B_T-log F coordinate system before use of the finite differences method; spectrum B was produced by smoothing the B_T data in this coordinate system with the cubic spline algorithm before transforamtion to the affinity distribution. Both spectra have been normalized by the total binding site concentration (B_{MAX}) estimated by the regression displayed in figure 4-15. Spectrum A (equivalent to the regression of figure 4-15): equilibrium dissociation constant K_d = 8.8 nM; spectrum B (data smoothed by "model-free" cubic spline): equilibrium dissociation constant K_d = 5.3 nM. Method of finite differences: input spacing log K = 0.1, log a = 0.2. N(K) is dimensionless; dimensionality of K is M^{-1} (liters/mole). Inset: Total binding (B_T) data of figure 4-8 smoothed by cubic spline algorithm in the log B_T-log F coordinate system (B_T data points not shown). The subroutine uses a statistical criterion to smooth the data, but there is no force-fit to a specific model as in the regression shown in figure 4-15. The "spline-smoothed" binding curve was transformed into spectrum B by the method of finite differences (Thakur et al., 1980).

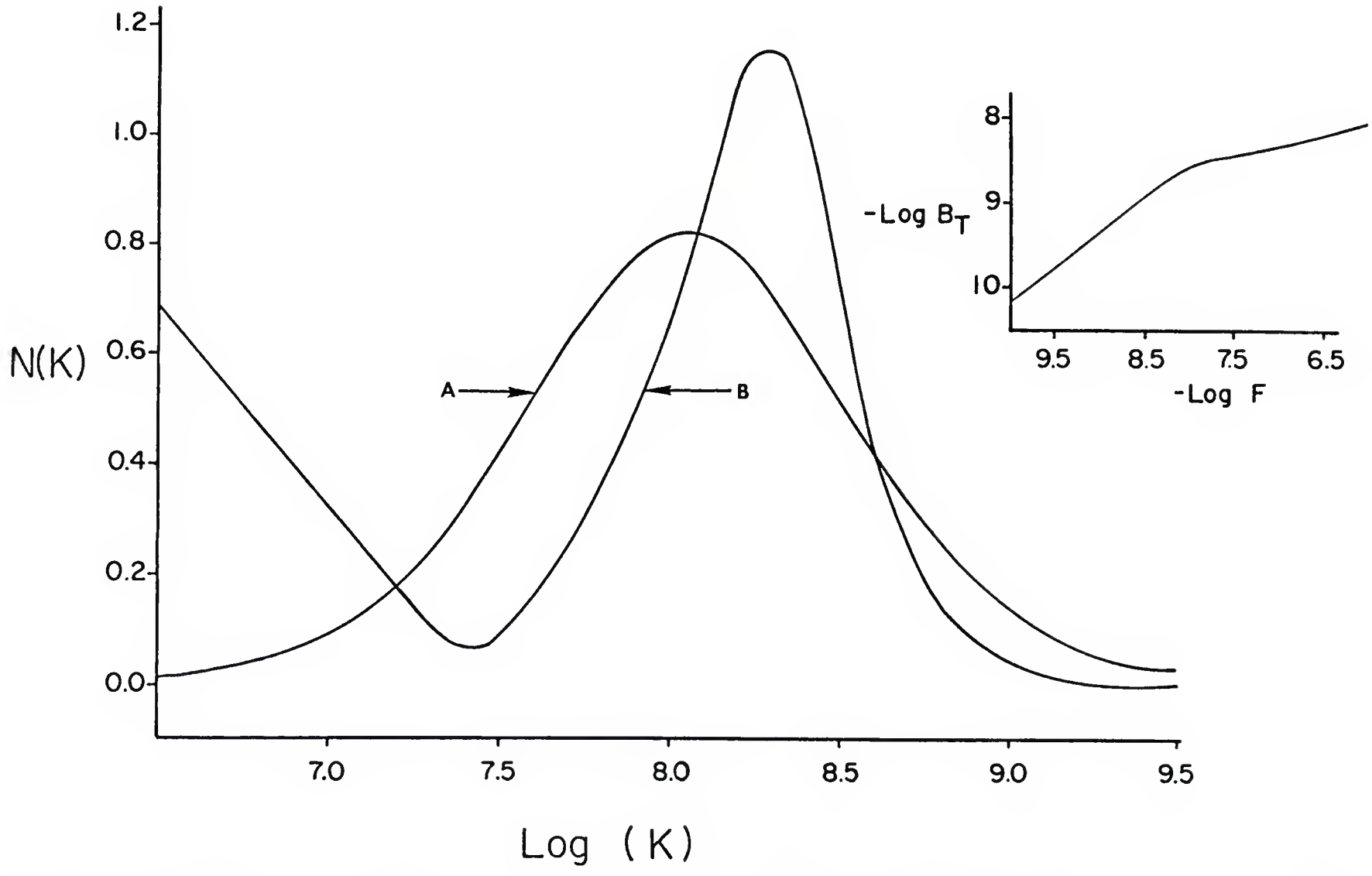

1.2
1.0
0.8
0.6
0.4
0.2
0.0
N(K)
A
B
7.0
7.5
8.0
8.5
9.0
9.5
Log (K)
8
9
10
-Log B_T
9.5
8.5
7.5
6.5
-Log F

Fig. 4-24. Merged affinity spectral analysis of the binding of [^{3}H]dexamethasone to glucocorticoid receptors in perfused mouse brain cytosol (derived from the values of total binding, B_T, from 18 independent experiments combined with the individual estimates, for each experiment, of the receptor concentration, B_0, derived from initial Scatchard plots such as figure 4-10).

Affinity distribution A is a transformation of normalized total binding data "predicted" by the 2-parameter nonlinear regression displayed as the curve in figure 4-16 (i.e., binding data that have been "smoothed" by force-fit to a specific binding model); thus, spectrum A is the representation of a single homogeneous class of high-affinity binding sites. Distribution B is a transformation of total binding data that have been smoothed by the cubic spline algorithm described in Methods. In both cases the transformation was performed on binding functions expressed in the log (B_T/B_0) - log F coordinate system. Spectrum A was generated by transforming the regression curve of figure 4-16 to the log (B_T/B_0) - log F coordinate system before application of the method of finite differences; spectrum B was produced by smoothing the (B_T/B_0) data in this coordinate system with the cubic spline algorithm before transformation to the affinity distribution. Spectrum A (equivalent to the regression of figure 4-16): equilibrium dissociation constant K_d = 1.8 nM; spectrum B (data smoothed by "model-free" cubic spline): equilibrium dissociation constant K_d = 1.7 nM. Method of finite differences: input spacing log K = 0.1, log a = 0.2. N(K) is dimensionless; the dimensionality of K is M^{-1} (liters/mole). Inset: normalized total binding (B_T/B_0) data smoothed by cubic spline algorithm in the log (B_T/B_0) - log F coordinate system (data points not shown). The subroutine uses a statistical criterion to smooth the data, but the data are not constrained to fit any specific biophysical model as in the regression of figure 4-16. The "spline-smoothed" binding curve was transformed into spectrum B by the method of finite differences (Thakur et al., 1980).

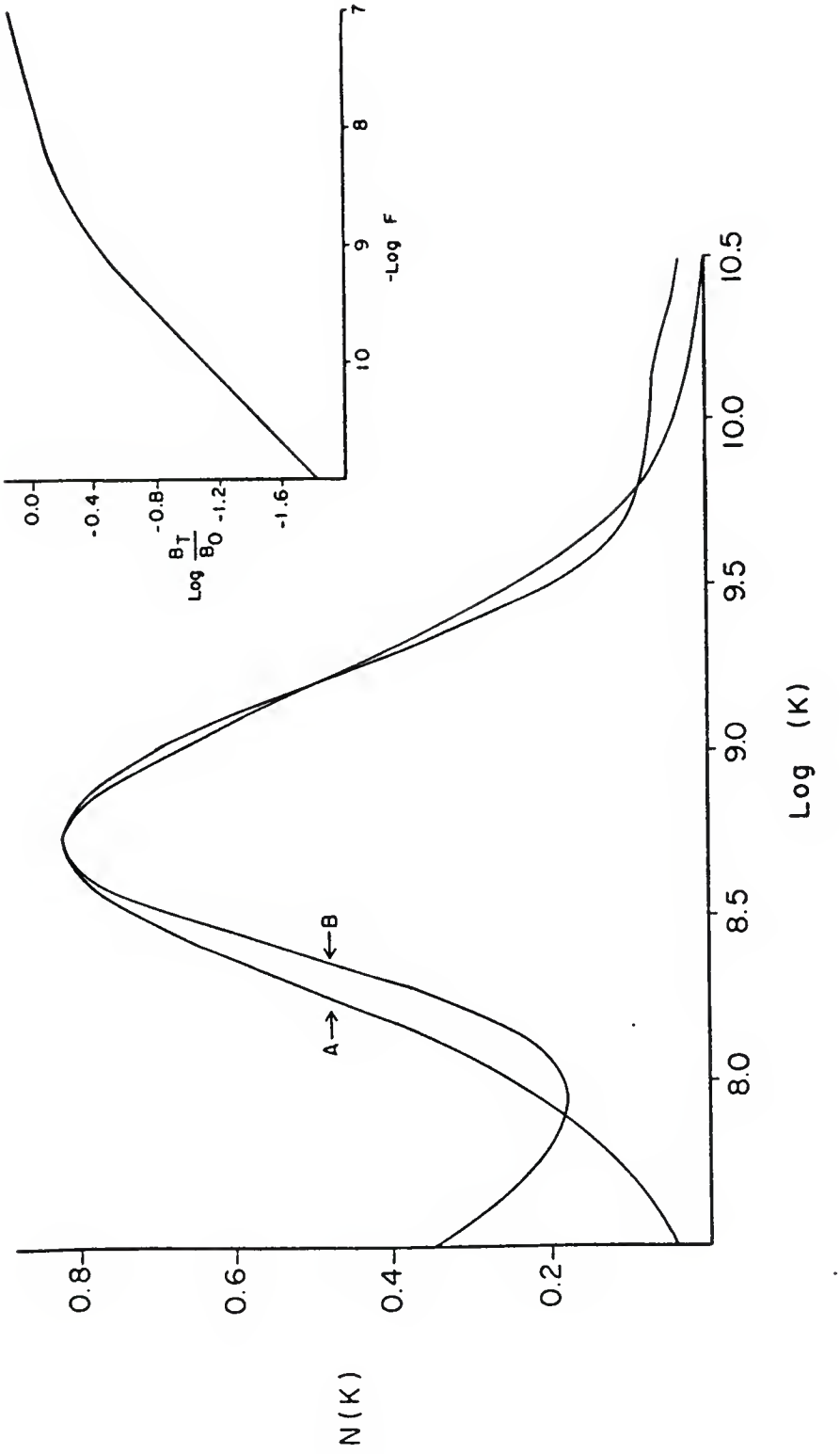
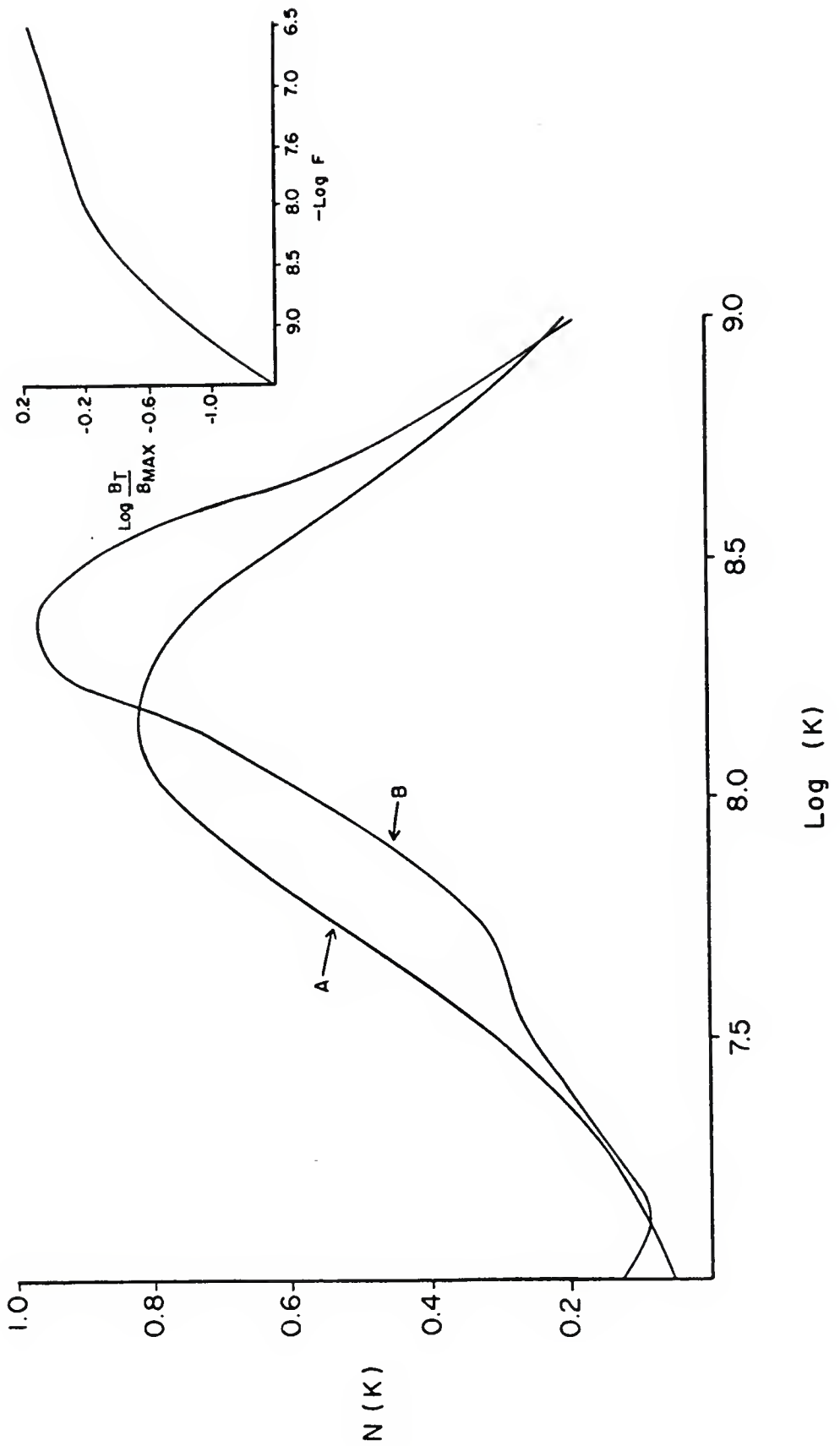

N(K)
0.8
0.6
0.4
0.2
A→
←B
8.0
8.5
9.0
9.5
10.0
10.5
Log (K)
Log $\frac{B_T}{B_O}$
0.0
-0.4
-0.8
-1.2
-1.6
10
9
8
7
-Log F

Fig. 4-25. Merged affinity spectral analysis of the binding of [^{3}H]corticosterone to the complete ensemble of corticosterone binding sites in perfused mouse brain cytosol (derived from the values of total binding, B_T, from 7 independent experiments combined with the individual estimates, for each experiment, of the total binding site concentration, B_{MAX}, derived from initial Scatchard plots such as figure 4-12).

Affinity distribution A is a transformation of normalized total binding data "predicted" by the 2-parameter nonlinear regression displayed as the curve in figure 4-17 (i.e., binding data that have been "smoothed" by force-fit to a specific binding model); thus, spectrum A is the representation of a single homogeneous class of high-affinity binding sites. Distribution B is a transformation of total binding data that have been smoothed by the cubic spline algorithm described in Methods. In both cases the transformation was performed on binding functions expressed in the log (B_T/B_{MAX}) - log F coordinate system. Spectrum A was generated by transforming the regression curve of figure 4-17 to the log (B_T/B_{MAX}) - log F coordinate system before application of the method of finite differences; spectrum B was produced by smoothing the (B_T/B_{MAX}) data in this coordinate system with the cubic spline algorithm before transformation to the affinity distribution. Spectrum A (equivalent to the regression of figure 4-17): equilibrium dissociation constant K_d = 6.6 nM; spectrum B (data smoothed by "model-free" cubic spline): equilibrium dissociation constant K_d = 4.6 nM. Method of finite differences: input spacing log K = 0.1, log a = 0.2. N(K) is dimensionless; the dimensionality of K is M^{-1} (liters/mole). Inset: normalized total binding (B_T/B_{MAX}) data smoothed by cubic spline algorithm in the log (B_T/B_{MAX}) - log F coordinate system (data points not shown). The subroutine uses a statistical criterion to smooth the data, which are not constrained to fit a specific biophysical model as in the regression of figure 4-17. The "spline-smoothed" binding curve was transformed into spectrum B by the method of finite differences (Thakur et al., 1980).


1.0
0.8
0.6
0.4
0.2
N (K)
7.5
8.0
8.5
9.0
Log (K)
A
B
0.2
-0.2
-0.6
-1.0
Log $\frac{B_T}{B_{MAX}}$
9.0
8.5
8.0
7.6
7.0
6.5
-Log F

[^{3}H]dexamethasone and [^{3}H]TA to glucocorticoid receptors in perfused mouse brain cytosol. Both plots are consistent with a single population of non-cooperative, high-affinity glucocorticoid binding sites (for which [^{3}H]TA has higher affinity than [^{3}H]dexamethasone), and both suggest similar estimates of the total receptor concentration (B_0).

Figure 4-12 (derived from the specific binding data of figure 4-8) is a conventional plot of the binding of [^{3}H]corticosterone to the complete ensemble of saturable corticosterone binding sites in perfused brain cytosol; deceptively, it is also consistent with a single population of binding sites, but the resulting estimate of binding site concentration (B_{MAX}) is significantly greater than estimates obtained with [^{3}H]dexamethasone and [^{3}H]TA. Since it seemed likely that this large ensemble of corticosterone binding sites contained a subset pool of contaminating plasma transcortin (CBG) or intracellular CBG-like binding sites that were distinct from the actual glucocorticoid receptors, separate [^{3}H]corticosterone isotherms were also generated by incubating one of each pair of cytosol samples with a 200-fold excess of unlabeled dexamethasone (instead of unlabeled corticosterone). With this substitution the "nonspecific" correction (B_{NS}) increased dramatically as expected (not shown), limiting the "specific" binding B_{sp} to the sites which bound both [^{3}H]corticosterone and dexamethasone. The plot of such an isotherm is shown in figure 4-13; it also is consistent with a single class of non-cooperative binding sites (i.e., putative glucocorticoid receptors). (The apparent difference between the K_d estimates generated by figures 4-12 and 4-13 is not reproducible, nor is the apparent difference between the B_0 estimates generated by figures 4-10 or 4-11 and figure 4-13; see summary table 4-1.) The

absence of evidence for a second class of lower affinity binding sites (i.e., the lack of negative curvature or apparent negative cooperativity) in experiments such as that of figure 4-12 thus suggested that [^{3}H]corticosterone had similar affinity for both the putative glucocorticoid receptors and the non-receptor binding sites (i.e., the [^{3}H]corticosterone sites having very low affinity for dexamethasone). The characteristics of the "nonglucocorticoid", CBG-like binding sites were explored further, and will be discussed in chapter V.

In order to test the validity of binding parameter estimates derived from conventional Scatchard plots fit by simple least-squares linear regression, some isotherm data were analyzed by the weighted, 3-parameter nonlinear regression of total binding (B_T) on total [^{3}H]steroid concentration (S_L) as described in Methods. Figure 4-14 presents the results of such a reanalysis of the [^{3}H]dexamethasone experiment already depicted in figure 4-7 and analyzed conventionally in figure 4-10; and figure 4-15 depicts such a reanalysis of the [^{3}H]corticosterone binding data previously displayed in figure 4-8 and analyzed in figure 4-12. The complete regressions and the separate specific and nonspecific components of each were plotted in the usual Scatchard coordinate system, and agreement of the resulting nonlinear parameter estimates of K_d and B_0 (or B_{MAX}) with those derived from the conventional Scatchard plots (figures 4-10 and 4-12) was excellent.

Several methods of combining the data from different individual isotherms to generate a single "merged" estimate of K_d were used to analyze accumulated [^{3}H]dexamethasone and [^{3}H]corticosterone binding data from multiple experiments. Data sets were combined by the weighted, 2-parameter merged nonlinear regression of total binding (B_T)

on total [^{3}H]steroid concentration (S_L) as described in Methods, with insertion of the initial B_0 (or B_{MAX}) estimate for each experiment as an input variable, instead of as a parameter to be estimated for the complete ensemble of points. Figure 4-16 presents the results of such a merged analysis of 18 independent [^{3}H]dexamethasone binding isotherms, and figure 4-17 depicts such an analysis of 7 independent [^{3}H]corticosterone isotherms. The complete regressions and the separate specific and nonspecific components of each were plotted in the modified Scatchard coordinate system as shown; the agreement of the resulting nonlinear parameter estimates of K_d with the means of the individual K_d estimates derived from conventional Scatchard plots was excellent (see table 4-1).

Data sets were also combined in merged Hill plots (i.e., plots of log $[B_{sp}/(B_0-B_{sp})]$ or logit [θ, fractional occupancy] vs. log F) that were allowed to span the 20%-80% occupancy range. In such plots the lines were fit by the polynomial cubic spline subroutine described in Methods; this analysis provided both quantitiative descriptions of the shapes of the Hill plots at medium values of occupancy and merged estimates of the dissociation constants (K_d). Figure 4-18 is such a combined plot containing points from 18 independent [^{3}H]dexamethasone binding isotherms, and figure 4-19 presents a Hill plot of points derived from 7 independent [^{3}H]corticosterone isotherms. The "regression" lines in both figures 4-18 and 4-19 are nearly linear, and both Hill coefficients (0.99 and 0.95, the instantaneous slopes of the regression lines at the 50% occupancy level) were close to 1.0, suggesting that the binding sites do not interact. Agreement of estimates of K_d derived from these merged plots with the means of

conventional Scatchard-derived estimates was good (see table 4-1), and the merged Hill coefficients confirmed the absence of cooperativity suggested by Hill plots of individual experiments fit by linear regression over a wide range of occupancy.

Data sets were also merged in simple unweighted "free receptor" plots (fit by 2-parameter least-squares linear regression as described in Methods, although only the slope estimates were of interest). Figure 4-20 is a combined "free receptor" plot containing points from the 18 independent [^{3}H]dexamethasone isotherms, and figure 4-21 presents such a reanalysis of the points derived from the 7 independent [^{3}H]corticosterone isotherms. Both regression lines passed near the origin as expected, and the resulting estimates of K_d were in agreement with the estimates generated by the other methods.

An attempt was made to analyze individual isotherm data sets by transforming the total binding (B_T) data to continuous affinity distributions or spectra as described in Methods; the results were disappointing for individual experiments containing relatively few data points. Figure 4-22 presents such a spectral analysis of the [^{3}H]dexamethasone experiment depicted in figure 4-7 and analyzed also in figures 4-10 and 4-14; and figure 4-23 depicts spectra derived from the [^{3}H]corticosterone binding data previously displayed in figure 4-8 and analyzed again in figures 4-12 and 4-15. In each figure the inset shows the "smoothed" binding data that were transformed to the "unconstrained" spectra (labeled "B"); the comparison spectra ("A") were transformed from the 3-parameter nonlinear regression curves shown in figures 4-14 and 4-15 and are characteristic of a single class of noncooperative, saturable binding sites. The shapes, widths, locations, and even the

numbers of peaks in the unconstrained ("B") spectra shown in figures 4-22 and 4-23 are not truly "representative". These characteristics were quite variable within a series of independent replications, reflecting the extreme sensitivity of the affinity distributions to moderate levels of experimental error. For example, the suggestions of apparent positive cooperativity (peak sharpening and high-affinity shift with respect to the comparison spectrum) and the rather bizarre hints of saturable low-affinity (nonspecific) binding displayed in figures 4-22 and 4-23 were not reproducible; other experiments that generated very similar Scatchard plots yielded affinity spectra possessing very different characteristics (e.g., broad, multimodal, low affinity-shifted peaks suggestive of site heterogeneity or apparent negative cooperativity). Individual isotherms simply did not contain enough data points to permit useful application of the method, which is very sensitive to the slight changes in the shape of the spline-smoothed binding curves that are produced by different patterns of error variance.

The affinity distribution analysis proved rather more useful when it was applied to larger sets of data points formed by merging independent isotherms (as described in Methods). Figure 4-24 is such a spectral analysis of data from 18 independent [^{3}H]dexamethasone binding isotherms (127 points), and figure 4-25 presents the analysis of 50 points from 7 independent [^{3}H]corticosterone isotherms. In each figure the inset shows the "smoothed" normalized binding data (B_T/B_0) that were transformed to the unconstrained spectra ("B"); the comparison spectra ("A") were transformed from the 2-parameter merged nonlinear regression curves that are shown in figures 4-16 and 4-17 and that are each

indicative of a single saturable site. The unconstrained [^{3}H]corticosterone spectrum "B" (figure 4-25, derived from 50 points) was still irregular and did not closely match the comparison spectrum "A"; although the complete ensemble of [^{3}H]corticosterone binding sites contains at least two distinct subclasses (see Chapter V), the peculiar shape and high-affinity shift of the peak were probably spurious, and we do not consider them statistically significant. The high-affinity region of the [^{3}H]dexamethasone spectrum "B", however, could be superimposed upon its comparison spectrum "A" (figure 4-24). The large input data set (127 points) and the sensitivity of the method to subleties in the shape of the binding curve strengthened the conclusion that the glucocorticoid receptors constituted a homogeneous class of noninteracting sites, and the center of the "B" peak provided an additional, independent estimate of the dissociation constant K_d.

The measurements of equilibrium binding parameters for the three [^{3}H]ligands are summarized in table 4-1. The different methods of analyzing the [^{3}H]dexamethasone and [^{3}H]corticosterone isotherms described above generated consistent estimates of K_d for the binding of [^{3}H]dexamethasone to putative glucocorticoid receptors (first column of results) and (with the exception of the irregular [^{3}H]corticosterone merged affinity spectrum showing a high-affinity shift, method G) for binding of [^{3}H]corticosterone to the larger ensemble of all its binding sites (last column). Because the data sets for the merged analyses were derived from multiple independent experiments, the "asymptotic" standard errors (indicated by the b superscript) are not sufficiently conservative and should not be used as inferential statistics; they are presented merely for comparisons among the different combined methods.

Reanalysis of the three experiments performed initially to measure the efficiency of the DEAE filter assay (method B: incubation of a constant concentration of [^{3}H]dexamethasone with samples of increasing cytosol concentration) also generated a consistent estimate of K_d for [^{3}H]dexamethasone. Method D was merely a modification of the merged nonlinear regression (method C) in which B_{sp} (instead of B_T) was an input variable and the nonspecific term (containing the parameter C_1) was neglected. As expected, the affinity of [^{3}H]TA for the putative receptors (second column) was significantly higher (at least 6-fold) than that of [^{3}H]dexamethasone. The significantly lower affinity of [^{3}H]corticosterone for the putative receptors (third column) was not different from the combined affinity of [^{3}H]corticosterone for the complete ensemble of its binding sites (last column).

The ratios of measured rate constants (described in chapter VI) also provided estimates of the dissociation constants for interactions of the three ligands with the putative glucocorticoid receptors; these estimates of K_d were included in the table (method H) for comparison with the equilibrium results. The equilibrium and kinetic estimates of K_d for [^{3}H]corticosterone (third column) were in reasonable agreement. In the case of each high-affinity synthetic ligand the measured equilibrium dissociation constant was larger than the ratio of the dissociation and association rate constants (k_d/k_a); this discrepancy was 8-fold for [^{3}H]TA and 4-fold for [^{3}H]dexamethasone. Although large, these discrepancies were considerably smaller than those previously reported to arise from studies of the binding of glucocorticoids to cytosol preparations from other target tissues (e.g., Pratt et al., 1975; Yeakley et al., 1980). The reduction or elimination of the

inactivation of unoccupied receptor sites in vitro achieved by the inclusion of molybdate and other protective buffer ingredients has probably contributed to the decrease in this discrepancy observed in our experiments; the cause or causes of the residual disagreement are unknown.

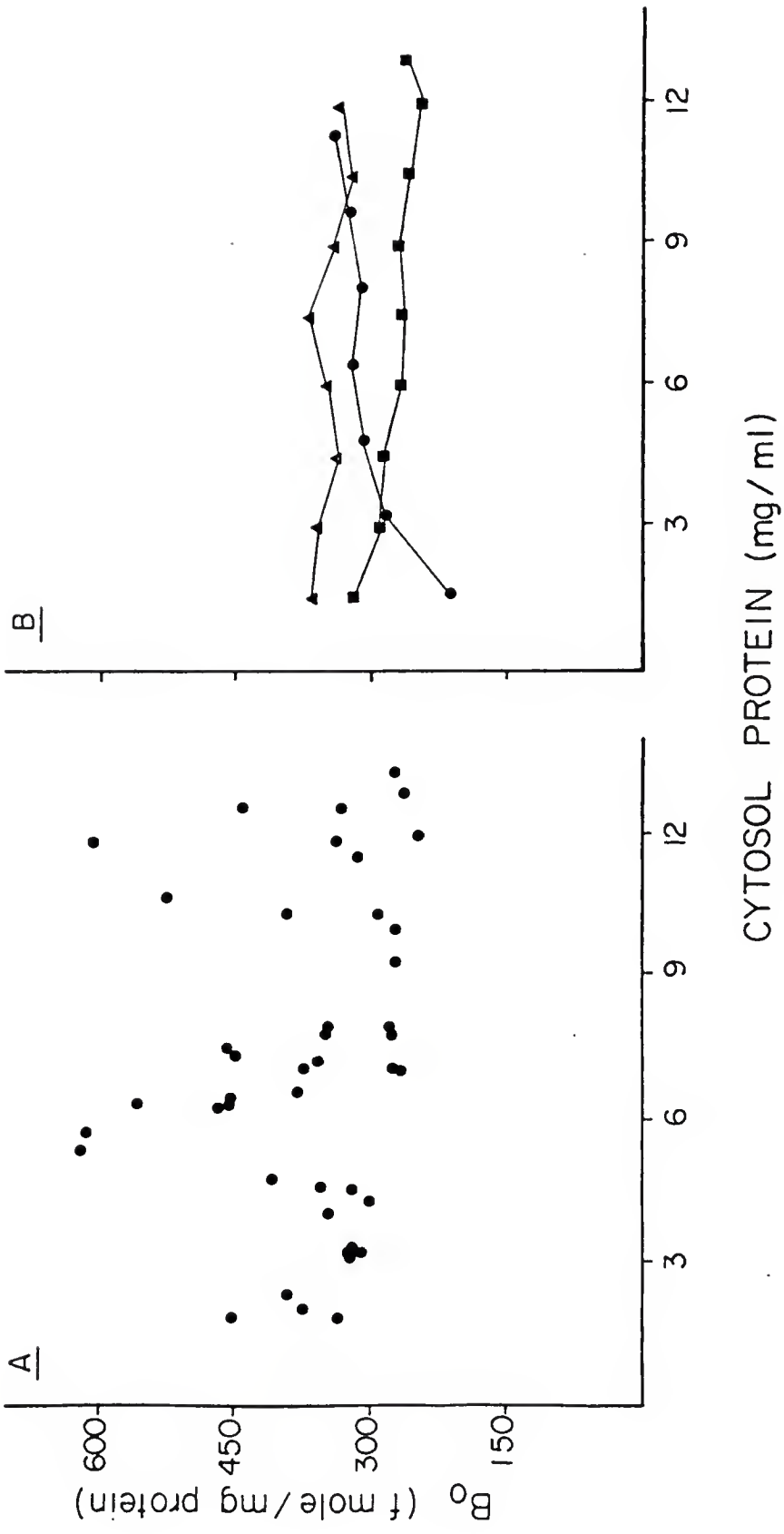
As expected, all Hill coefficients (methods I and F in table 4-1) were not different from the value of 1.0 that suggests noninteracting binding sites.

The measured functional binding site concentrations (B_0 and B_{MAX}) for the three [^{3}H]ligands are also summarized in table 4-1. The concentrations of putative glucocorticoid receptors measured with the different ligands (first three columns) were in excellent agreement, and these putative receptors comprised 63% of the complete ensemble of [^{3}H]corticosterone binding sites (column 4), which included a class of CBG-like sites that lack affinity for [^{3}H]dexamethasone and [^{3}H]TA (see chapter V).

The total receptor concentration (B_0) was not consistently or significantly affected either by the tissue: buffer ratio at homogenization or by dilution of the cytosol with additional buffer following homogenization. The left panel of figure 4-26 presents the estimated receptor concentrations and the corresponding cytosol protein concentrations for the independent [^{3}H]dexamethasone experiments. The plot reveals the wide range of receptor concentrations estimated by the different experiments; however, the slope of a regression line through the points was not different from 0. (The slope of a regression line through the points of a plot of estimated K_d vs. cytosol protein concentration for the different [^{3}H]dexamethasone isotherms was also

Fig. 4-26. Left panel: effect of cytosol protein concentration immediately following homogenization (produced by varying the tissue:buffer A ratio) on the measured total glucocorticoid receptor concentration (B_0) in perfused mouse brain cytosol.

The results of 43 independent experiments are plotted in the figure. Measurements of maximal specific binding were performed either by Scatchard analysis (n=18) or with single ligand concentration (saturation with 2 x 10^{-8} M [^{3}H]dexamethasone) assays (n=25). Experimental conditions are described in the legend to figure 4-7 and in Methods. The slope of a linear regression through these points is not different from 0. Right panel: The effect of dilution with buffer A following homogenization on the total receptor concentration (B_0) in perfused mouse brain cytosol. Three different pools of concentrated cytosol were diluted as shown to 8 different concentrations and then divided into pairs of samples. Following incubation for 12 h at 2°C with 2 x 10^{-8}M [^{3}H]dexamethasone and, for one of each sample pair, a 200-fold excess of unlabeled dexamethasone, 50 µl aliquots were assayed in triplicate by the DEAE filter assay for the determination of specific binding. Total receptor concentration (B_0) was not significantly affected by dilution of the cytosol following homogenization.


A
B
B_o (f mole/mg protein)
600
450
300
150
3
6
9
12
CYTOSOL PROTEIN (mg/ml)

not different from 0; not shown). Furthermore, the right panel of figure 4-26 shows, for 3 independent pools of concentrated cytosol, that dilution of initially concentrated cytosols with additional buffer did not exert a significant effect on the subsequently measured concentration of functional binding sites.

The two different methods used to calculate the equilibrium dissociation constants (K_{di}) for the binding of competing unlabeled ligands to [^{3}H]dexamethasone binding sites are illustrated in figure 4-27. The figure presents a representative two-ligand competition "free-receptor" plot used to determine K_{di} for the binding of unlabeled corticosterone to putative receptors labeled with [^{3}H]dexamethasone in perfused brain cytosol; the reciprocal of the slope provided an estimate of K_{di}. The points were derived (as described in Methods and in chapter III) from the specific binding data of the two isotherms displayed in the Scatchard plots in the left inset. These data were generated by the "Edsall-Wyman" experimental design: the isotherm described by the steeper line was generated without unlabeled corticosterone, and the isotherm described by the plot having the reduced slope was generated in a similar fashion with the same pool of cytosol, except that each pair of tubes contained additionally the same concentration (8 nM) of unlabeled corticosterone. The K_{di} was also calculated directly from the slopes of these Scatchard plots (left inset, figure 4-27) by use of the familiar Edsall-Wyman equation as described previously. Figure 4-28 shows a representative merged competition "free receptor" plot used to determine the dissociation constant (K_{di}) for the binding of unlabeled progesterone (P_4) to putative receptors labeled with [^{3}H]dexamethasone. The points were derived from 4 independent experiments that each

Fig. 4-27. Representative two-ligand competition "free receptor" plot used to determine the equilibrium dissociation constant (K_{di}) for the binding of unlabeled corticosterone (B) to glucocorticoid receptors labeled with [^{3}H]dexamethasone in perfused mouse brain cytosol.

The points were derived from the data displayed in the Scatchard coordinate system in the left inset. The nomenclature of the figure conforms to that of chapter III, with the identification of L (labeled ligand) with [^{3}H]dexamethasone and C (competing ligand) with corticosterone (B). The plot is described in the text and in detail in chapter III. The abscissa was calculated by equation (4-9); equation (4-9) was also used, in conjunction with the known corticosterone concentration (S_C), to find the ordinate, which is the bound/free ratio for the competing corticosterone. The line (equation 4-11) was fit by simple linear regression and was not constrained to intersect the orgin. The reciprocal of its slope is the equilibrium dissociation constant (K_{di} or K_{dC}) for corticosterone: K_{di} = 5.2 nM. Right inset: the abscissa of the plot described above has been changed from total free receptor to total bound receptor concentration in order to display the range of receptor occupancy (θ) spanned by the experimental conditions; range of θ: 67% to 96%. Left inset: Representative experimental data (generated by the "Edsall-Wyman" experimental design and displayed in the Scatchard coordinate system) used to determine the dissociation constant for corticosterone (K_{di}) by competition with labeled [^{3}H]dexamethasone for glucocorticoid receptors. The upper isotherm (-B,●) was generated as described in the text and in the legend to figure 4-7, and the lower curve (+8 nM B,▲) was generated in an identical fashion with the same pool of cytosol, except that both members of each pair of tubes contained 8 nM unlabeled corticosterone to reduce the slope of the "Scatchard" plot as depicted. The lines were fit by simple linear regression, and the [^{3}H]dexamethasone dissociation constant K_{dL} and receptor concentration B_0 were derived from the slope and intercept of the upper [^{3}H]dexamethasone Scatchard plot (●). These values of K_{dL} and B_0 were used, in conjunction with the reduced slope of the lower plot (▲), as input to equation (4-8) to calculate the K_{di} for corticosterone (see text and also chapter III). The data (▲) were also transformed as described above into the competition "free receptor" plot displayed in the figure. For the specific experiment displayed: K_{dL} = 2.3 nM, B_0 = 2.5 nM (270 fmole/mg protein); the protein concentration was 9.3 mg/ml, and the corticosterone K_{di} = 5.9 nM (by use of the Edsall-Wyman equation).

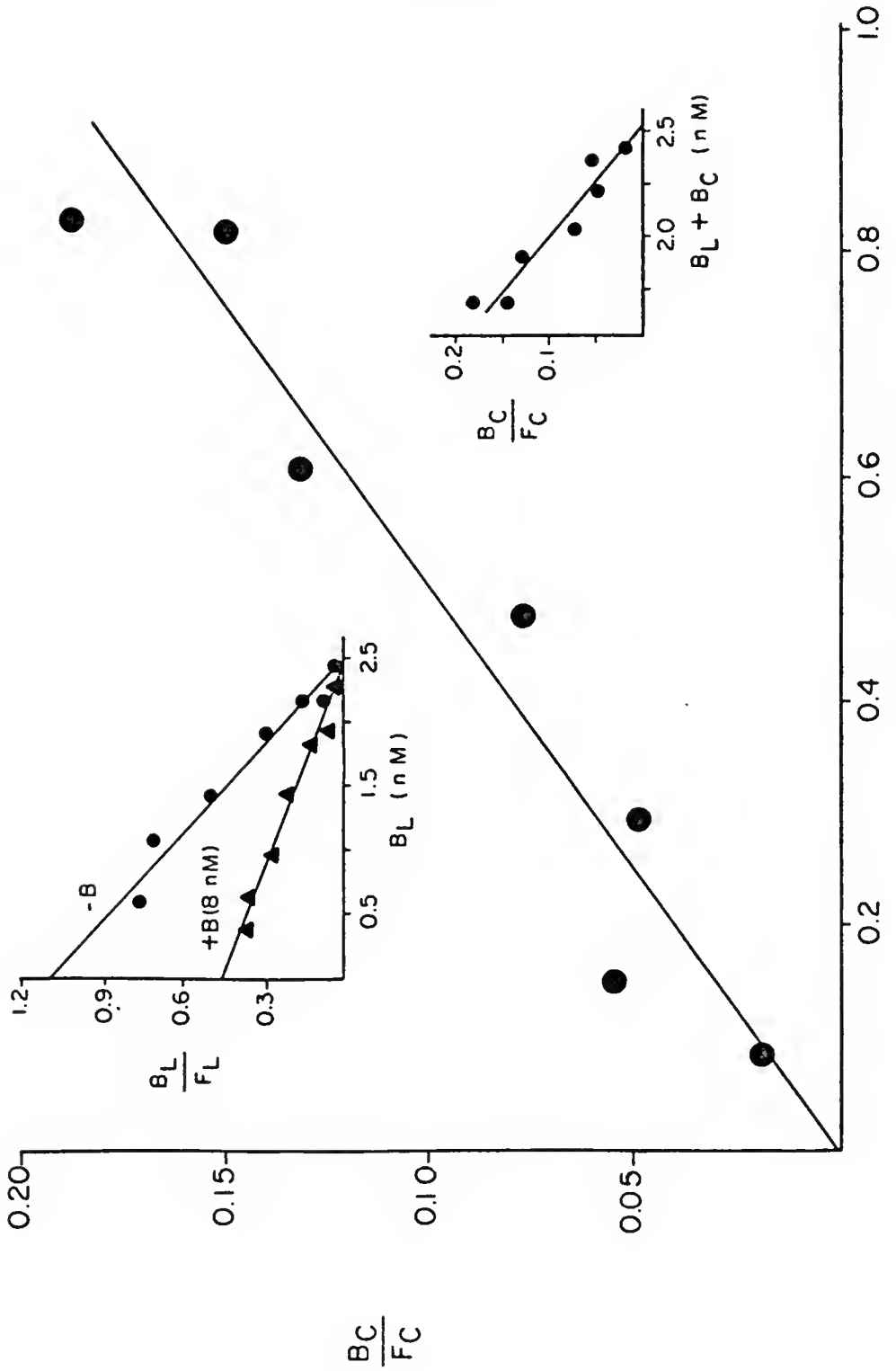
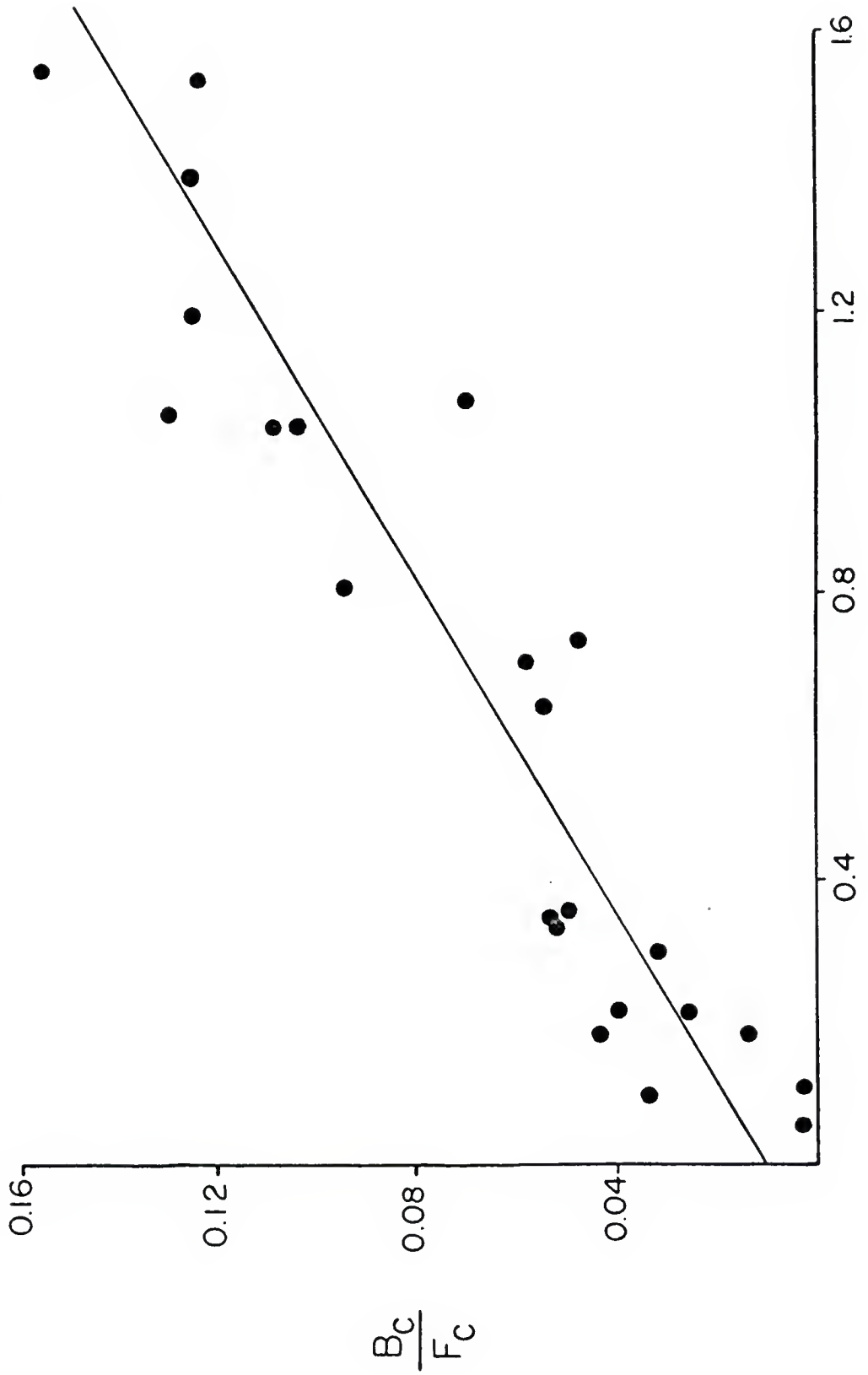

0.20
0.15
0.10
0.05
$\frac{B_C}{F_C}$
0.2
0.4
0.6
0.8
1.0
$B_O - B_L - B_C$ (nM)
1.2
0.9
0.6
0.3
$\frac{B_L}{F_L}$
-B
+B(8 nM)
0.5
1.5
2.5
B_L (nM)
0.2
0.1
$\frac{B_C}{F_C}$
2.0
2.5
$B_L + B_C$ (nM)

Fig. 4-28. Merged competition "free receptor" plot used to determine the equilibrium dissociation constant (K_{di}) for the binding of unlabeled progesterone (P_4) to glucocorticoid receptors labeled with [^{3}H]dexamethasone in perfused mouse brain cytosol.

The nomenclature of the figure conforms to that of chapter III, with the identification of L (labeled ligand) with [^{3}H]dexamethasone and C (competing ligand) with progesterone. The points were derived from 4 independent experiments that employed the "Edsall-Wyman" experimental design. For each independent experiment a pair of isotherms was generated as described in the text and in the legends to figures 4-7 and 4-27. One of the [^{3}H]dexamethasone isotherms was generated in the absence of competing progesterone; the other isotherm was generated in the presence of a constant concentration of unlabeled progesterone, which reduced the apparent affinity of the binding sites for the [^{3}H]dexamethasone. The [^{3}H]dexamethasone equilibrium dissociation constant K_{dL} and receptor concentration B_0 were derived from a Scatchard analysis of the isotherm generated in the absence of progesterone. These values of K_{dL} and B_0 were used, in conjunction with the competition data from the tubes containing progesterone, to derive the points displayed in the plot, which is described in the text and discussed in detail in chapter III. The abscissa was calculated by equation (4-9); equation (4-9) was also used, in conjunction with the known progesterone concentration (S_C), to find the ordinate, which is the bound/free ratio for the competing progesterone. The line (described by equation 4-11) was fit by linear regression and was not constrained to intersect the origin. The reciprocal of the slope is the equilibrium dissociation constant (K_{di} or K_{dC}) for progesterone: K_{di} = 11.8 ± 1.0 nM (n=4). The progesterone concentrations for the 4 experiments were: 10, 10, 16 and 52 nM; the range of protein concentration: 5.3-10.0 mg/ml. Use of the nonlinear regression program to generate a standard error for the slope of the line is described in Methods.


0.16
0.12
0.08
0.04
$\frac{B_C}{F_C}$
0.4
0.8
1.2
1.6
$B_O - B_L - B_C$ (nM)

employed the Edsall-Wyman experimental design. Since the free receptor plot estimated only one binding parameter (K_{di}), the points from the for different experiments (which contained both different protein and unlabeled progesterone concentrations) could be combined before the regression (which was not constrained to pass through the origin) was performed.

The results of the competition experiments are summarized in table 4-2, which presents the estimates of the equilibrium dissociation constants (K_{di}) for the binding of selected steroids to the putative receptors labeled by [^{3}H]dexamethasone. The estimates of K_{di} were calculated for each individual experiment both by the free receptor method and by use of the Edsall-Wyman relation; the individual data sets for each competitor were also combined by the merged free receptor method. Additionally, the measured affinity of unlabeled dexamethasone for the receptor subset of the complete ensemble of [^{3}H]corticosterone binding sites (described in Chapter V) was also included in the table (first entry). Values of K_{di} calculated by the Edsall-Wyman relation were in excellent agreement with those derived from the free receptor plots, and the merged estimates were not different from the means of the individual determinations.

Based strictly upon their ability to stimulate the activity of tyrosine aminotransferase (TAT) in hepatoma (HTC) cells, these steroids (see table 4-2 for definitions of abbreviations) have been classified (e.g., Rousseau and Schmit, 1977) as either optimal inducers (o: DEX, B, F, ALDO), suboptimal inducers (s: DOC), or antiglucocorticoids (a: P_4, S, T); our experiments did not include steroids having truly negligible affinity for the receptors (inactive steroids such as THB,

Table 4-2. Equilibrium dissociation constants (K_{di} values) for the binding of selected steroids to the glucocorticoid receptors of perfused mouse brain cytosol. Tabulated values are calculated from the same experimental data by three different methods as shown; see text and also chapter III for details. The labeled ligand displaced by the competitors (with the indicated exception) is [^{3}H]DEX. Values are $\bar{x}$ ± s.e.m., with the number of determinations given in parentheses.

STEROID[a]	"FREE RECEPTOR" PLOTS: MEANS OF INDIVIDUAL EXPERIMENTS	MERGED "FREE RECEPTOR" PLOT	"EDSALL-WYMAN" EQUATION: MEANS OF INDIVIDUAL EXPERIMENTS
	($\times 10^{-9}$M)	($\times 10^{-9}$M)	($\times 10^{-9}$M)
DEX/[^{3}H]B[b]	1.0 ± 0.2 (6)	1.0 ± 0.1[c] (6)	Not Applicable
B	4.7 ± 0.6 (4)	4.7 ± 0.5[c] (4)	5.3 ± 0.6 (4)
DOC	8.5 ± 2.5 (4)	8.9 ± 1.5[c] (4)	9.0 ± 2.3 (4)
P_4	12.6 ± 1.9 (4)	11.8 ± 1.0[c] (4)	12.2 ± 2.0 (4)
F	13.1 ± 0.9 (4)	16.6 ± 2.5[c] (4)	13.4 ± 1.1 (4)
ALDO	27.0 ± 3.5 (4)	27.1 ± 3.2[c] (4)	27.0 ± 3.3 (4)
S(DOF)	51.4 ± 4.1 (3)	45.7 ± 10.8[c] (3)	42.1 ± 2.0 (3)
T	591 ± 62 (3)	616 ± 110[c] (3)	536 ± 60 (3)

a. Definitions of abbreviations for trivial names of steroids: DEX = dexamethasone, B = corticosterone, DOC = deoxycorticosterone, F = cortisol, ALDO = aldosterone, S(DOF) = cortexolone (deoxycortisol),

Continued

Table 4-2. (Continued)

T=testosterone.

b. Measured affinity of dexamethasone for the receptor subset of the total pool of [^{3}H]corticosterone binding sites. See text and chapter V.

c. "Asymptotic" s.e.m. generated by the nonlinear regression program (SAS).

tetrahydrocorticosterone). The ligands fell into the following order of decreasing affinity (increasing K_{di}) for the [^{3}H]dexamethasone binding sites: (DEX, o) > B (o) > DOC (s) > P_4 (a) $\simeq$ F (o) > ALDO (o) > S (a) > T (a), where the hepatoma TAT-inducer type (o, s, or a) has been included in parentheses. The steroids of a given inducer category were not clustered perfectly in the affinity rank order. Thus, the TAT optimal inducer aldosterone had relatively low affinity, and the antigluco- corticoid progesterone (P_4) was as effective in competition as the familiar glucocorticoid cortisol (F). The measured affinity of progesterone (P_4) for the mouse brain glucocorticoid receptors (K_{di}=13 nM) was much higher than the value (K_{di}=52 nM) reported for the hepatoma (HTC) cytosol [^{3}H]dexamethasone binding sites (Rousseau and Schmit, 1977). Unlabeled dexamethasone had high affinity for the receptor subset of the total pool of [^{3}H]corticosterone binding sites as expected.

Physiochemical Characteristics of Mouse Brain Glucocorticoid Receptors

The size and potential heterogeneity of the mouse brain cytosol glucocorticoid receptor labelled with the nearly-irreversible agonist [^{3}H]TA were studied by gel filtration through Sephacryl S-300 in buffer A containing additionally 150 mM KCl as described in Methods. Figure 4-29 presents a representative S-300 profile that displays the in vitro stabilization by molybdate and other ingredients of buffer A of a well-resolved and relatively large (77 Å) [^{3}H]TA-receptor complex. Figure 4-30 illustrates calibration of the S-300 column. The sharpness of the included peak and the moderate ionic strength afforded by 150 mM

Fig. 4-29. In vitro stabilization and molecular size of the mouse brain glucocorticoid receptor during incubation and Sephacryl S-300 chromatography in buffer A (containing additional 150 mM KCl).

Cytosol prepared in buffer A + 150 mM KCl was incubated at 2°C for 12-16 h with 10 nM [^{3}H]TA (●) or 10 nM [^{3}H]TA and a 200-fold excess of unlabeled TA (■). Following incubation, free [^{3}H]TA was removed by gel filtration in the same buffer on a large (1.5 x 98 cm) Sephadex G-25 (fine) column. Samples from the void volume of the G-25 column (2 ml, approximately 5 mg/ml) were then chromatographed on a column of Sephacryl S-300 superfine (1.5 x 98 cm) at 12 ml/h in the same buffer (A + 150 mM KCl). Fractions (2 ml) were collected, and 1 ml aliquots of these were assayed for radioactivity. Distribution coefficients (K_d) were calculated from elution volumes (V_e), the void volume (V_o) indicated by blue dextran, and the total liquid volume (V_i) marked by [^{14}C]antipyrene, [^{3}H]water and [2-^{3}H]deoxy(D)glucose: $K_d = (V_e - V_o)/(V_i - V_o)$. The column was calibrated with 7 standard proteins (chromatographed in separate runs as two different mixtures and detected by absorbance at 280 nm), and the Stokes radius of the receptor complex (R_s) was calculated from regressions of R_s vs several different functions of the standard protein K_d values as shown in figure 4-30. The different standard curves agreed closely; for the specific experiment shown R_s = 76 Å. Mean and standard error from replicates are given in table 4-3.

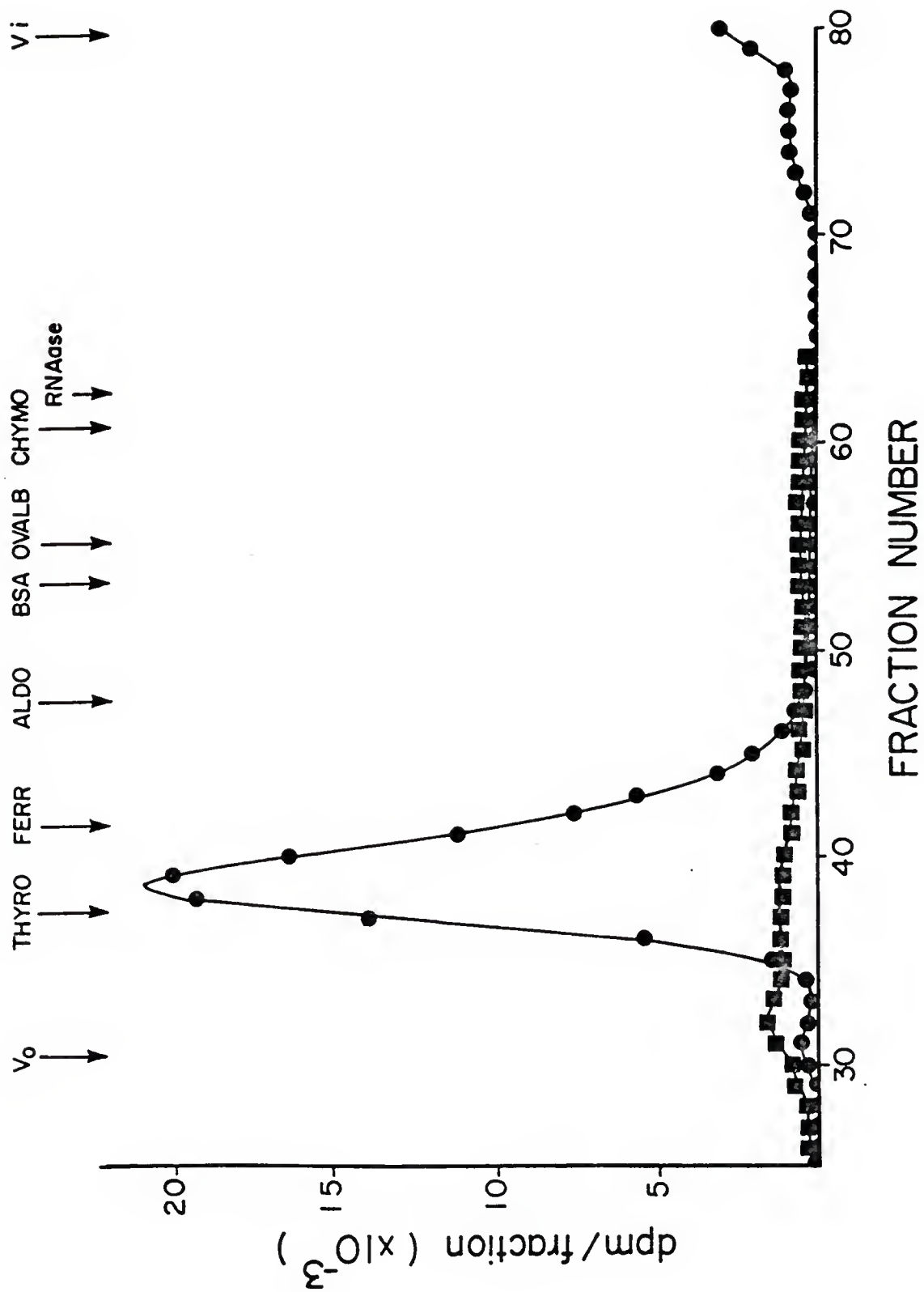
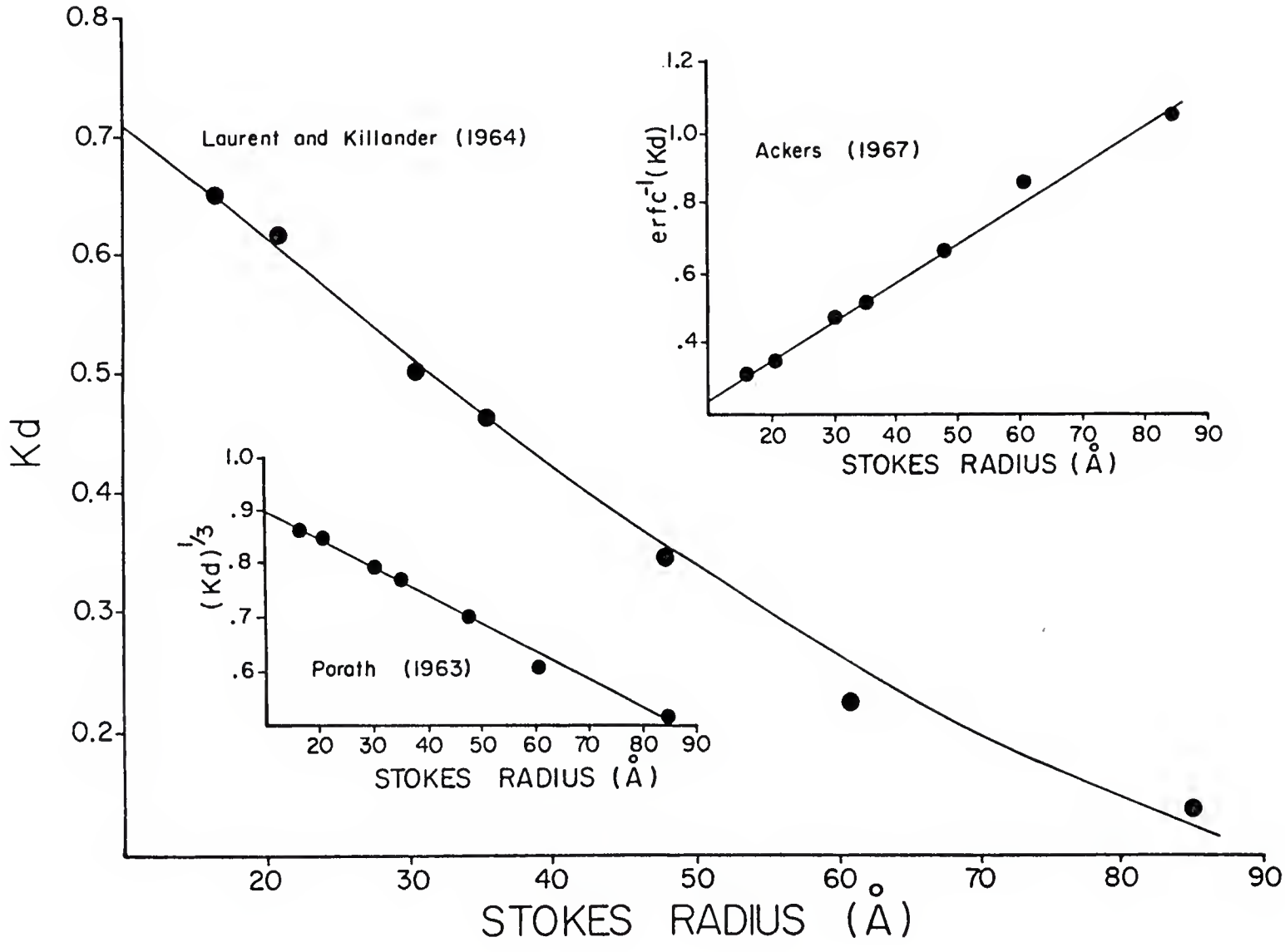

Vo
THYRO
FERR
ALDO
BSA
OVALB
CHYMO
RNAase
Vi
dpm/fraction (x10⁻³)
20
15
10
5
30
40
50
60
70
80
FRACTION NUMBER

Fig. 4-30. Calibration of the Sephacryl S-300 column with standard proteins of known Stokes radius (R_s).

Samples (2 ml, in buffer A + .15 M KCl) of the standard proteins were run as the mixtures "A" or "B" described below. Column conditions are described in the legend to figure 4-29. Mixture "A": blue dextran, void volume (V_o); ferritin, 61 Å; aldolase, 48.1 Å; ovalbumin, 30.5 Å; RNAase, 16.4 Å; [^{3}H]2-deoxy(D)glucose, total liquid volume (V_i). Mixture "B": thyroglobulin, 85 Å; bovine serum albumin, 35.5 Å; chymotrypsinogen A, 20.9 Å; [^{14}C]antipyrene or [^{3}H]water, total liquid volume (V_i). Fractions were assayed by absorbance at 280 nm. Distribution coefficients K_d were calculated as described in the text and in the legend to figure 4-29. Standard curves were generated by the linear methods of Porath (1963) and Ackers (1967), and by the nonlinear regression of Laurent and Killander (1964). The three methods of regression are in close agreement. In the method of Ackers (1967) the ordinate y is defined by the relation $K_d = 1 - \mathrm{erf}(y)$, where erf(y) is the standard error function derived from the normal distribution. In the method of Laurent and Killander (1964) the data are fit by the equation $K_d = \exp[-C_1(R_s + C_2)^2]$, where C_1 and C_2 are the adjustable parameters of the regression. This method also may be linearized (not shown) by plotting the Stokes radius R_s vs $(-\ln K_d)^{1/2}$ for the standard proteins.


Laurent and Killander (1964)
Kd
STOKES RADIUS (Å)
Ackers (1967)
erfc⁻¹(Kd)
STOKES RADIUS (Å)
Porath (1963)
(Kd)^1/3
STOKES RADIUS (Å)

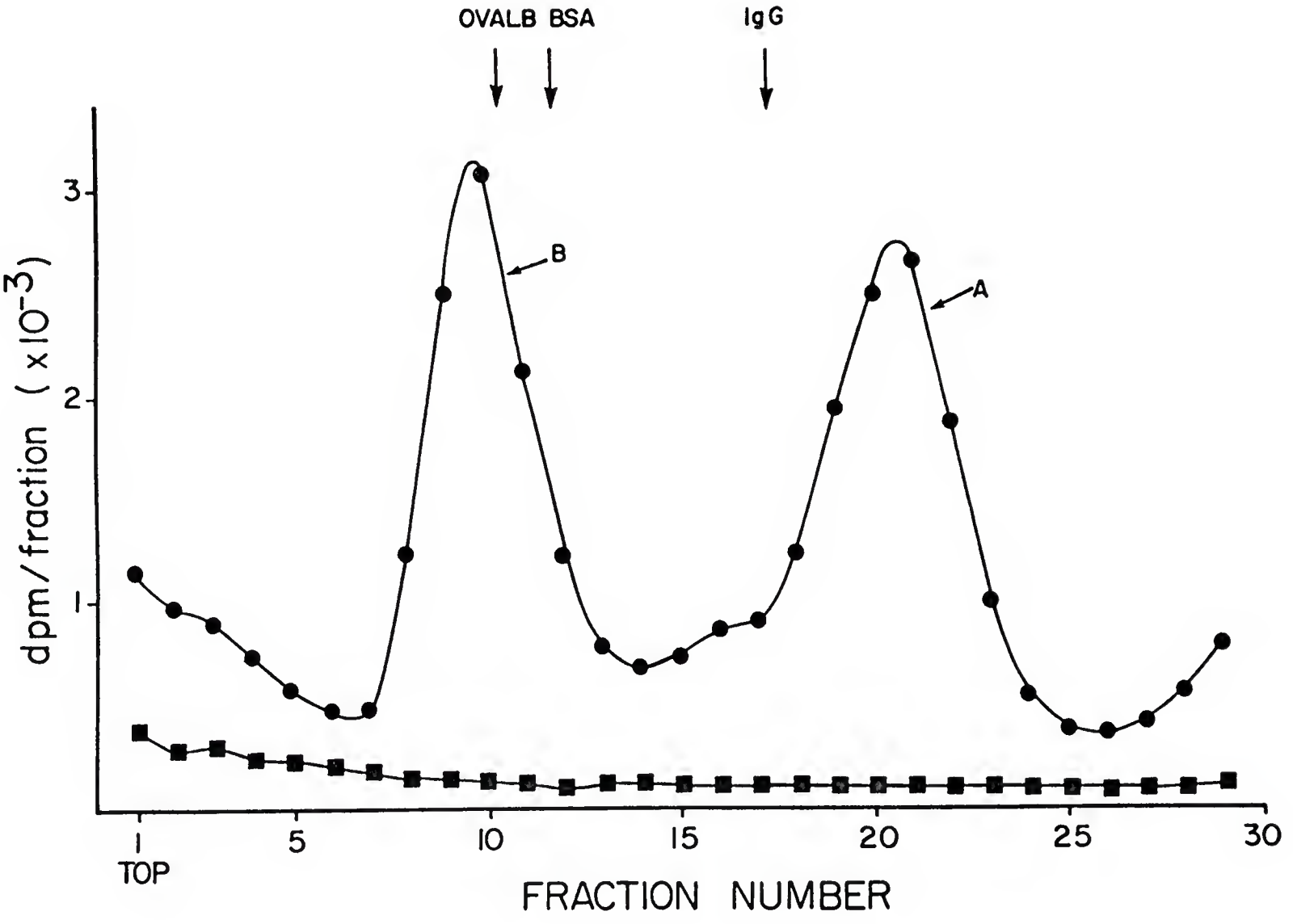
KCl in the buffer suggested that although it could possibly be for oligomeric, the large hormone-receptor complex was not merely an artifactual aggregate formed by homogenization in hypotonic medium. The peak was not, however, completely symmetrical; it had a small shoulder at about 65 Å that accounted for approximately 15% of the total area. The presence of 150 mM KCl was not required for the resolution of the major peak, nor was the peak signficantly shifted or altered by the addition of 350 mM KCl to the basic buffer A (not shown). In the absence of molybdate the S-300 profile was influenced by the ionic strength and was not completely reproducible, frequently revealing minor peaks having smaller Stokes radii (not shown). The exact locations and the precise conditions leading to the probable proteolytic formation of these secondary peaks were not studied further, but they may be related to proteolytic fragments of glucocorticoid receptors observed previously that retained functional steroid binding sites (e.g., Sherman, Pickering, Rollwagen and Miller, 1978; Wrange and Gustafsson, 1978). Although the complete profile was not reproducible in the absence of molybdate, the major peak was resolved well at 59 ± 1 Å (n=8) in buffer containing 150 mM KCl, and the slight shoulder observed with molybdate (e.g., figure 4-29) was absent; this result is consistent with previous measurements (performed without molybdate) of 57-61 Å for the radii of [^{3}H]TA- and [^{3}H]dexamethasone- receptor complexes extracted from rat, mouse and human tissues (e.g., Stevens and Stevens, 1979; Wrange, Carlstedt-Duke and Gustafsson, 1979). In hypotonic buffer (without molybdate) the 59 Å peak was shifted forward toward the position of the large species observed in the presence of molybdate. The relation of the large complex observed in the presence of molybdate to the smaller

one found in its absence is not known, but it is possible that signficant activation (transformation) occurred at moderate ionic strength in the absence of molybdate (e.g., Milgrom, Atger and Baulieu, 1973). Thus, the different Stokes radii may be a consequence of activation; this possibility requires further investigation by measurement of the Stokes radius of the activated receptors in the presence of molybdate.

Following S-300 chromatography the sedimentation behavior of the partially purified [^{3}H]TA-receptor complex was investigated by centrifugation of the pooled, concentrated S-300 column fractions containing the receptor peak through glycerol gradients (made up in buffer A containing 150 mM KCl) as described in Methods. Although the resulting sedimentation patterns were not completely reproducible, figure 4-31 presents a representative profile that displays the two well-resolved peaks that were typical of this experiment. The positions of the two peaks (A = 9.7 ± 0.4 S and B = 3.7 ± 0.1 S) were reasonably reproducible, but the ratio of the peak areas was quite variable. Furthermore, in some experiments one or the other of the two peaks was completely absent. Sedimentation of crude cytosol samples labelled with [^{3}H]TA (not partially purified by S-300) revealed two peaks at approximately the same locations, but they were not as well resolved as those described above; the crude cytosol profiles were heterodisperse, and some labelled material was found throughout the 3-12 S range (not shown). Attempts to measure the Stokes radius (by S-300 chromatography) of [^{3}H]TA-receptor complexes partially purified first by gradient sedimentation were disappointing; little bound radioactivity was recovered from the S-300 column. In summary, prolonged rate sedimentation of the

Fig. 4-31. Sedimentation of mouse brain glucocorticoid receptors in buffer A (containing additional 150 mM KCl) after partial purification by Sephacryl S-300 chromatography.

Following S-300 chromatography of cytosol labeled with [^{3}H]TA (●) or [^{3}H]TA and a 200-fold excess of unlabeled TA (■) (described in figure 4-29) aliquots (1 ml) from each of the 5 high fractions bracketing the peak of the S-300 profile (e.g., fractions 37-41 in figure 4-19) were pooled and concentrated 10-fold by ultrafiltration (Amicon Minicon B-15) to a volume of 500 µl. Aliquots (250 µl) of the concentrated, pooled S-300 fractions were then centrifuged at 2°C for 20 h at 234,000 g (average) through 5 ml gradients of 12.5 - 25% (w/v) glycerol in the same buffer (A + .15 M KCl). The cellulose nitrate tubes were punctured, and 175 µl fractions were collected and assayed for radioactivity. The value of $s_{20,w}$ for the indicated peaks A and B was calculated from the linear regression of $S_{20,w}$ vs sedimentation distance (Martin and Ames, 1961) for the standard proteins, which were run on parallel gradients as 250 µl samples in the same buffer (A + .15 M KCl): ovalbumin, 3.6 S; BSA, 4.3 S; IgG, 7.4 S. Standard proteins were labeled to low specific activity ([$^{14}CH_3$]-methylated) by the method of Rice and Means (1971). For the specific experiment depicted, the sedimentation coefficients were: peak A, 9.2 S; peak B, 3.3 S. Means and standard errors from replicates are presented in table 4-3.


OVALB
BSA
IgG
B
A
dpm/fraction (x10⁻³)
3
2
1
1
TOP
5
10
15
20
25
30
FRACTION NUMBER

partially-purified 77 Å [^{3}H]TA-receptor complex in buffer A supplemented with 150 mM KCl revealed two components that sedimented at 9.7 ± 0.4 S and 3.7 ± 0.1 S; the ratio of the peak areas was not reproducible.

The mean value of the receptor Stokes radius measured by S-300 chromatography and the mean sedimentation coefficients of the two peaks observed in the glycerol gradient profile are listed in table 4-3. Each sedimentation coefficient was combined with the single value of the Stokes radius and also with the assumed values of the partial specific volume and degree of hydration, in order to estimate the molecular weights, frictional ratios, and axial ratios that would result if either of the sedimentation peaks also possessed the measured Stokes radius. Apparently the molecular species resolved in the 9.7 S peak (A) corresponded to the 77 Å receptor complex; the degree of asymmetry estimated by assuming corespondence for the slower-sedimenting peak (B) was highly improbable. Thus, the molybdate-stabilized [^{3}H]TA-receptor complex has an apparent molecular weight of 315,000 daltons and is moderately asymmetric. The extent of receptor hydration (δ) is unknown; the value δ = 0.2 g H_2O/g protein was arbitrarily assumed in order to facilitate a direct comparison with the sizes and shapes reported by Sherman and her colleagues (e.g., Niu et al., 1981) for molybdate-stabilized steroid receptors. In hypotonic buffer containing 20 mM Na_2MoO_4, estrogen, progestin and glucocorticoid receptors from a variety of tissues all shared hydrodynamic parameters, molecular weights and shapes nearly identical to those listed in table 4-3 for the rapidly-sedimenting peak (A); Vedeckis (1981) also reported the value of 77 Å for the radius of glucocorticoid receptors from a mouse pituitary tumor cell line. These large, molybdate-stabilized [^{3}H]TA-receptor

Table 4-3. Physical properties of the molybdate-stabilized mouse brain glucocorticoid receptors labeled with [^{3}H]TA. Values listed are, where appropriate, $\bar{x}$ ± s.e.m. The number of experiments is shown in parentheses. See figures 4-29 and 4-31 and "Methods" for details. A and B refer to the faster and slower-sedimenting peaks observed in the centrifugation experiments. The tabulated calculations arbitrarily assume that peaks A and B both correspond to the same value of R_S (see text for discussion).

Parameter	Gradient Peak A	Gradient Peak B
R_S ($\times 10^{-8}$ cm)	77 ± 2 (10)	77 ± 2 (10)
$S_{20,w}$ ($\times 10^{-13}$ sec^{-1})	9.7 ± 0.4 (4)	3.7 ± 0.1 (7)
M (daltons)	315,000 ± 20,000	120,000 ± 5000
f/fo (δ=0)	1.70	2.34
f/fo (δ=0.2 g/g)	1.57	2.16
b/a (δ=0)	13:1 (prolate)	29:1 (prolate)
b/a (δ=0.2 g/g)	10:1 (prolate)	24:1 (prolate)

complexes may be oligomeric, since the molecular weights of purified for receptor polypeptides are much smaller than 315,000 daltons (e.g., Govindan and Sekeris, 1978; Wrange, Carlstedt-Duke and Gustafsson, 1979; Nordeen, Lan, Showers and Baxter, 1981).

Four of the five immobilized-dye columns included in the Amicon Dyematrex kit retained large fractions of the total pool of [^{3}H]dexamethasone receptor complexes when dye-ligand screening was performed as described in Methods (data not shown). Red HE3B adsorbed the entire pool of receptors. Blue F3GA retained approximately 80%, and the Green A and Blue B columns bound about half the receptor pool. None of the potential "biospecific" affinity eluents released a significant amount of [^{3}H]dexamethasone from any column. Binding to the dyes was very tight; the high salt and urea steps were required for the release of [^{3}H]dexamethasone, which was complete only after the final treatment with 8 M urea and 0.5 M NaOH. The nature of the dye-receptor interaction is unknown, but hydrophobic interactions are believed to dominate the behavior of many proteins during dye chromatography (Dean and Watson, 1979). The results suggested that the receptors may possess surface hydrophic domains other than the steroid binding sites themselves, and that hydrophic interaction chromatography might prove helpful in a receptor purification scheme. The failure of some of the dye columns to adsorb the entire pool of steroid-receptor complexes requires further investigation and should not be considered strong evidence for receptor heterogeneity.

Discussion

We have found that perfused mouse brain cytosol contains a single, apparently homogeneous class of saturable, high-affinity binding sites for the synthetic glucocorticoid agonists [^{3}H]dexamethasone and [^{3}H]TA; these binding sites possess the steroid specificity and other generally-accepted characteristics of putative glucocorticoid receptors. The mouse brain is probably fundamentally similar to the rat brain in possessing additionally a low concentration of specific mineralocorticoid receptors that eventually become saturated in vitro at very high [^{3}H]dexamethasone concentrations; this inference is derived from observations of saturable high-affinity rat brain [^{3}H]aldosterone binding sites from which [^{3}H]aldosterone is displaced by high concentrations of dexamethasone (Anderson and Fanestil, 1976; Moguilewsky and Raynaud, 1980; Veldhuis, van Koppen, van Ittersum and de Kloet, 1982). However, these rat brain mineralocorticoid binding sites have never been revealed by Scatchard analysis as a second class of lower-affinity, saturable [^{3}H]dexamethasone binding sites, nor have the experiments reported here disclosed a second class of saturable mouse brain [^{3}H]dexamethasone binding sites. Furthermore, the concentration of putative glucocorticoid binding sites measured with [^{3}H]TA (which possesses very low mineralocorticoid activity) was not less than the estimate obtained with [^{3}H]dexamethasone. Thus, we do not believe that mineralocorticoid sites have been inadvertently confounded with glucocorticoid receptors in any of the experiments reported here.

Perfused mouse brain cytosol also contains a class of corticosterone binding sites that lack affinity for the synthetic glucocorticoids dexamethasone and TA; these sites have many of the

properties of transcortin (CBG). Although it has not been established that these binding sites are intracellular in vivo, their concentration in plasma may not be sufficient to explain their presence in perfused brain cytosol as a contaminant derived from residual blood remaining in the brain after perfusion (see Chapter V). These sites have not, however, been observed in perfused rat hippocampal cytosol (e.g., Wrange, 1979; Veldhuis et al., 1982).

Subcellular fractionation in the hypertonic medium (buffer A) revealed negligible concentrations of high-affinity [^{3}H]dexamethasone binding sites in nuclear, mitochondrial, and microsomal fractions. This finding does not preclude the existence of membrane-associated glucocorticoid binding sites differing from the soluble sites in steroid affinity and specificity. Rat brain synaptic plasma membranes have been reported to contain saturable (but very low affinity) sites that bind corticosterone, dexamethasone and TA (Towle and Sze, 1978). Other, CBG-like plasma membrane-associated glucocorticoid binding sites lacking affinity for dexamethasone and TA have also been reported (e.g., Koch, Lutz-Bucher, Briaud and Mialhe, 1978; Harrison, Balasubramanian, Yeakley, Fant, Svec and Fairfield, 1979; Harrison, Yeakley and Fant, 1980). Such "unusual" binding sites would not have been detected in our experiments that employed only a single, moderate concentration (2×10^{-8} M) of [^{3}H]dexamethasone to assay the concentration of specific binding sites in each subcellular fraction; the possibility that mouse brain contains membrane-associated binding sites will require further investigation. Furthermore, the in vivo subcellular localization of the soluble binding sites is unknown. Subcellular redistribution of the unoccupied glucocorticoid binding sites may have occurred as a function

of the aqueous homogenization procedure, and future studies with non-aqueous nuclear isolation methods (e.g., Martin and Sheridan, 1980) may reveal significant nuclear concentrations of unoccupied soluble glucocorticoid binding sites.

The DEAE filter assay was convenient, reproducible and linear; it was used whenever the binding sites could be confined to the small volumes that can be absorbed by individual filter disks. It has been suggested that heat-activated hepatoma (HTC) cell glucocorticoid receptors may not bind to the Whatman DE-81 filter disks with high efficiency (Baxter, Santi and Rousseau, 1975); we have found very recently that this is also the case with activated mouse brain glucocorticoid receptors (Luttge and Gray, unpublished). This failure to bind strongly to DE-81 filters stands in contrast to the efficient binding of activated glucocorticoid receptors to DEAE-cellulose and DEAE Sephadex A-50 (e.g., Vedeckis, 1981; Parchman and Litwack, 1977). The two methods used to estimate the efficiency of the DEAE filter assay for the measurement of unactivated, molybdate-stabilized glucocorticoid receptors generated relatively high and equivalent estimates (76% ± 2%). Furthermore, the data presented in figure 4-4 suggest that the conductivity of buffer A is not dangerously close to the threshold value required for the elution from the DEAE filters of the molybdate-stabilized unactivated receptors that we have studied. Finally, we have noticed that much of the nonspecific binding observed with the filter assay is the result of direct adsorption of steroid by the filters, and thus is not related to the presence of the biological sample.

The requirement that a sulfhydryl-protective agent is necessary to preserve functional unoccupied glucocorticoid binding sites in mouse

brain cytosol is consistent with its requirement for the preservation of binding sites in cytosol prepared from thymocytes, lung, and spleen (Sando, Hammond, Stratford and Pratt, 1979; Granberg and Ballard, 1977), but contrasts strongly with the absence of such a DTT requirement in cytosol prepared from other tissues such as liver, kidney, heart, lymphocytes and fibroblasts (Granberg and Ballard, 1977; Wheeler, Leach, LaForest, O'Toole, Wagner and Pratt, 1981; Sando, LaForest and Pratt, 1979). This highly variable requirement for DTT may be a consequence of very different tissue concentrations of endogenous sulfhydryl protective compounds that contribute to the preservation of functional glucocorticoid binding site conformations in vivo by maintaining a favorable intracellular redox state (e.g., Granberg and Ballard, 1977).

The role of molybdate in the in vitro stabilization of unoccupied and also of nonactivated glucocorticoid receptor sites is considerably more mysterious than that of DTT in the preservation of functional unoccupied binding sites. Some molybdate effects may also be tissue dependent; for example, the ability of molybdate to convert activated heart cytosol glucocorticoid receptors back to the nonactivated form (Seleznev, Shnyra, Volkova, Smirnov, Djozdjevic-Markovic, Lan and Baxter, 1981) contrasts with its reported failure to reverse the activation of liver cytosol glucocorticoid receptors (Barnett et al., 1980) and with our very recent observation that it fails to reverse the activation of mouse brain glucocorticoid receptors (Luttge and Gray, unpublished). In any case, the importance of molybdate in the prevention of decapacitation cannot be overestimated. The decapacitation rate observed at 2°C in the absence of molybdate (figure 4-5) is high enough to prevent the accurate estimation of ligand

affinity from an isotherm generated by the prolonged (e.g., 16 h) incubation of cytosol samples, some of which contain low, subsaturating steroid concentrations; decapacitation of unoccupied receptors in the samples incubated with low concentrations of [^{3}H]steroid would lead to a serious overestimate of the dissociation constant (K_d). Thus, isotherms generated without molybdate may be distorted either by a failure to attain equilibrium (as a result of inadequate incubation time) in the samples containing low ligand concentrations or by the decapacitation of unoccupied binding sites at the low ligand concentrations; the results of such experiments (e.g., Veldhuis et al., 1982; Meyer and McEwen, 1982) must therefore be questioned.

In addition to protecting unoccupied binding sites, molybdate also facilitates the gradual conversion of some latent or "cryptic" binding sites to the functional steroid-binding form. Figure 4-5 reveals a signficant increase in brain cytosol binding sites as a function of cytosol "aging" (without steroid) in the presence of molybdate at 2°C, 12°C, and 22°C; this increase is consistent with the gradual molybdate-dependent conversion of latent to functional binding sites observed with thymocyte, lymphocyte, and liver cytosol preparations (Sando et al., 1979; Wheeler et al., 1981; Barnett et al., 1980). Many aspects of the in vitro up and down-regulation of glucocorticoid receptors are currently under investigation; at present none are well-characterized or understood. As examples, we have observed (in the absence of molybdate) that calcium ion promotes the decapacitation of functional glucocorticoid binding sites, that higher cytosol protein concentrations decrease the rate of decapacitation, and that components of a particulate subcellular fraction mediate an ATP-dependent reduction

in the rate of loss of binding sites (Luttge, Densmore and Gray, unpublished; Luttge et al., 1982).

The different methods of analyzing the equilibrium isotherm data produced, for a given [^{3}H]steroid, estimates of K_d that were in agreement (table 4-1); none of the merged methods generated an estimate of the [^{3}H]dexamethasone affinity that was different from the mean of the estimates derived from individual Scatchard plots (method A). None of the merged methods (i.e., C,D,E,F,G) was used to estimate B_0 (the total binding site concentration); the values of B_0 derived from initial Scatchard plots were input as variables so that B_0 could be eliminated from the set of adjustable regression parameters. The mean of the estimates derived from individual Scatchard plots (method A) and its relatively conservative standard error were chosen as the "best" estimate of K_d for comparison with reports from other laboratories. The methods (C and G) that included "nonspecific" binding in the regression model (i.e., that predicted total binding, B_T) generated estimates of K_d that were equivalent to those produced by the regressions (i.e., A,B,D,E,F) that predicted specific binding (B_{sp}). When derived from a large input data set, the merged affinity spectrum (method G) provided a sensitive test for apparent site cooperativity or heterogeneity.

Previous studies (e.g., Pratt et al., 1975; Yeakley et al., 1980) of glucocorticoid binding kinetics have consistently shown a lack of agreement between equilibrium and kinetic estimates of K_d; typically, the discrepancies have been highly correlated with the affinity of the ligand and are largest for the synthetic high-affinity agonists (TA and dexamethasone), but even the physiological steroid

[^{3}H]corticosterone usually has produced a significant (several-fold) discrepancy. For example, discrepancies of 79-fold, 12-fold, and 7-fold were reported for measurements with [^{3}H]TA, [^{3}H]dexamethasone, and [^{3}H]corticosterone, respectively, in pituitary tumor (AtT-20) cell cytosol (Yeakley et al., 1980). The discrepancies that we have observed are considerably smaller, but they remain significant for TA and dexamethasone. We cannot easily explain these discrepancies as artifacts resulting from the failure to reach equilibrium in the TA and dexamethasone isotherm experiments, since no increase in binding in the tubes containing low ligand concentrations was observed when incubations were extended beyond the reported times. Furthermore, in the standard buffer A the unoccupied sites were inactivated at a negligible rate; so that it is unlikely that an apparent equilibrium was merely a temporary steady state resulting from the combination of increasing receptor occupancy and a concomitant loss of unoccupied sites. Agreement of the equilibrium and kinetic estimates of K_d for the lower-affinity ligand corticosterone was reassuring, but an explanation for the residual discrepancies for dexamethasone and TA remains elusive.

We have not made a systematic effort to study the total glucocorticoid binding site concentration (B_0) as a function of the cytosol protein concentration; nor have we investigated, by systematically measuring B_0 as a function of the time after adrenalectomy, the possibility that adrenalectomy stimulates a gradual up-regulation of functional binding sites (e.g., Stevens et al., 1975). Although the slope of a regression line (not shown) fit to the plot (figure 4-26) of B_0 vs cytosol protein concentration was not different from 0, the range and standard deviation of B_0 were large. The source of this

variability is not obvious, since the pooling of brains to produce the cytosol for a specific experiment should have reduced the effect of animal individual differences on the standard deviation of the independent estimates of B_0. Although the result was not surprising, the absence of a correlation between B_0 and cytosol protein concentration has not been a universal finding; dilution experiments with cytosol derived from other tissue sources have sometimes revealed a strong effect of cytosol protein on the concentration of functional sites (sometimes attributed to the presence of an endogenous modulator of the steroid binding sites; e.g., Katsumata, Baker and Goldman, 1981).

The measured specificity of the [^{3}H]dexamethasone binding sites (table 4-2) was consistent with that expected of putative glucocorticoid receptors. The specificity of the mouse brain receptors may be compared with the following sequences of decreasing steroid affinity (increasing K_{di}) for [^{3}H]dexamethasone or [^{3}H]TA binding sites in hepatoma (HTC) cell cytosol, rat hippocampal cytosol, and chick thymus cytosol: (DEX) > B > DOC $\simeq$ F > ALDO > P_4 > S > T, hepatoma cells (Rousseau and Schmit, 1977); DOC $\simeq$ B > P_4 $\simeq$ DEX > ALDO, rat hippocampus (Veldhuis et al., 1982); and (DEX) > DOC $\simeq$ P_4 > B $\simeq$ F > ALDO > S > T, chick thymus (Aranyi, 1982, measured by a kinetic method developed recently). The affinity sequence F > ALDO > S > T has been found consistently by all investigators; the relative affinities of B, DOC, and F, however, have not always been in agreement. Consistent with the aforementioned measurements of steroid affinity for glucocorticoid receptors in chick thymus cytosol (Aranyi, 1982) and rat hippcampal cytosol (Veldhuis et al., 1982), we have found that progesterone has high affinity for the putative mouse brain receptors; progesterone possessed a significantly

lower affinity for the hepatoma (HTC) cytosol glucocorticoid receptors (Rousseau and Schmit, 1977). Although it is difficult to predict intracellular steroid concentrations from measured plasma concentrations, and although in vivo (37°C) steroid affinities have not been measured, the observed in vitro affinity of P_4 for the mouse brain glucocorticoid receptor sites is probably high enough to assure their saturation by the extremely high levels of the circulating antiglucocorticoid P_4 (360 nM) found in the pregnant mouse (Murr, Stabelfeldt, Bradford and Geschwind, 1974). The possible physiological consequences of a lengthy blockade of receptor-mediated glucocorticoid mechanisms during pregnancy are unknown. The observation that corticosterone has higher affinity for the rat hippocampal [^{3}H]dexamethasone binding sites than unlabeled dexamethasone itself (Veldhuis et al., 1982) is without precedent in the glucocorticoid literature and stands in contrast to our finding that unlabeled dexamethasone possesses high affinity for the putative receptor subset of the ensemble of whole mouse brain [^{3}H]corticosterone binding sites (table 4-2).

The competitor dissociation constants (K_{di} values) calculated by the "free receptor" method were in agreement with those calculated with the Edsall-Wyman relation describing the effect of a constant concentration of a competitive inhibitor; as expected, the K_{di} values for the higher affinity competitors B and DOC calculated by the free receptor method were slightly lower (indicating higher affinity) than those calculated with the Edsall-Wyman equation, but these differences were not significant. The values of K_{di} for the binding of unlabeled dexamethasone to the receptor subset of the [^{3}H]corticosterone binding sites and for the binding of unlabeled corticosterone to the

[^{3}H]dexamethasone binding sites were smaller (indicating higher affinity) than the K_{di} values estimated directly with, respectively, [^{3}H]dexamethasone and [^{3}H]corticosterone. These discrepancies may have resulted from the possible failure of the complex two-ligand mixtures to attain complete equilibrium. It is relevant that a continuous increase with time in steroid K_{di} values for mammary gland glucocorticoid binding sites labeled with [^{3}H]dexamethasone (measured by the competitive displacement "ED_{50}" method at 0°C) was observed when incubations were prolonged up to 44 h, well beyond the time required for the attainment of equilibrium in the absence of the unlabeled inhibitors (Weisz, Hutchens and Markland, 1982). The times required for such two-ligand mixtures to approach equilibrium depend on the steroid kinetic parameters and cannot be estimated easily, even if all kinetic parameters are known; such a simulation would require the numerical integration of the two simultaneous binding rate equations (see chapter II). Thus, some steroid competition K_{di} experiments may require extremely long incubations; in the future it will be necessary to verify empirically that a steady state has been attained for each ligand concentration in such an experiment. Most published steroid K_{di} measurements may thus be underestimates of the "true" equilibrium values.

It is interesting that the size and shape of the large, non-activated, molybdate-stabilized mouse brain [^{3}H]TA-receptor complex (table 4-3, peak "A") are identical to those of molybdate-stabilized estrogen, progestin and glucocorticoid receptors from a variety of tissue sources (e.g., Hutchens, Markland and Hawkins, 1981; Niu et al., 1981; Vedeckis, 1981). A single recent observation has indicated that

the large 9S receptor forms may actually exist in vivo and are not merely molybdate-generated artifacts: very rapid ($\underline{\sim}$ 2 h) sucrose gradient sedimentation of cytosol progesterone receptors in a vertical rotor without molybdate produced a single, sharp receptor peak at 9 S, suggesting that the native receptor form may be a large complex that is unstable in vitro and that requires molybdate for its preservation during lengthy ($\underline{\sim}$ 20 h) conventional gradient sedimentation runs (Grody et al., 1982). It seems paradoxical that steroid receptors composed of quite distinct polypeptides may form, in the presence of molybdate (and possibly in vivo), oligomeric complexes sharing nearly identical hydrodynamic parameters; for example the progesterone receptor consists of two distinct polypeptide subunits (A = 79,000 daltons and B = 108,000 daltons; e.g., Grody et al., 1982), whereas glucocorticoid receptors purified from several different tissues have been found to contain a single polypeptide (A = 87,000 daltons; e.g., Nordeen et al., 1981). However, the tetrameric structures that have been proposed for the native (in vivo) and molybdate-stabilized nonactivated receptor forms, such as A_2B_2 (374,000 daltons) for the progesterone receptor and A_4 (348,000 daltons) for the glucocorticoid receptor could possess very similar sizes and shapes that cannot be distinguished by simple hydrodynamic measurements. Since estimates of molecular weight derived from SDS-PAGE and from hydrodynamic parameters do not always agree exactly, the SDS-PAGE molecular weights listed above for hypothetical tetrameric complexes are not inconsistent with the hydrodynamic estimates of 315,000 daltons that we and others have observed.

What is the nature of the slowly-sedimenting (3.7 S) peak "B" (figure 4-31, table 4-3) that is produced by prolonged sedimentation of

the partially-purified 77Å steroid-receptor complexes in the continued presence of 10 mM Na_2MoO_4? Since we have postulated that the large (77Å, 9-10 S) complex is a tetramer composed of identical subunits, the 3.7 S peak could represent the partial conversion of the 77Å tetramer into the smaller monomers. This proposal is strengthened by our very recent discovery that the presence of 20 mM molybdate (instead of the nominal 10 mM concentration in buffer A) during the sedimentation of [^{3}H]TA-receptor complexes in crude cytosol stabilized the 9-10 S peak A and completely eliminated the slowly-sedimenting peak B, suggesting that the partial conversion of peak A (9-10 S) to peak B (3.7 S) previously observed in 10 mM molybdate was probably not a result of proteolytic cleavage of the large complex. (The 77Å radius of the large complex was not altered by the higher molybdate concentration.) In fact, we have not detected in the presence of molybdate any of the receptor fragments produced by limited proteolysis and retaining functional steroid binding sites that have been observed in the absence of molybdate by others (e.g., Sherman et al., 1978; Wrange and Gustafsson, 1978).

Very recently we have also examined the physiochemical properties of heat-activated (transformed) mouse brain [^{3}H]TA-receptor complexes in cytosol that has been adjusted to 20 mM Na_2MoO_4 following warming to room temperature (22°C) for a period of 45 min (Luttge and Gray, unpublished). The activated [^{3}H]TA-receptor complexes were sedimented in gradients containing 20 mM Na_2MoO_4, or gel filtered on a column of Sephcryl S-300 equilibrated with buffer contianing 20 mM Na_2MoO_4. The nucleophilic activation of the [^{3}H]TA-receptor complexes effected by the warming procedure, and the failure of subsequently added 20 mM molybdate to reverse the activation of receptors warmed in its absence, were

confirmed by observing the increased binding of activated receptor complexes to DNA-cellulose (by a modification of standard methods; e.g., Dahmer et al., 1981). In the presence of molybdate the activated receptors sedimented at 3.7 S and possessed Stokes radius 58 Å; the single well-resolved S-300 and gradient peaks (not shown) were symmetrical and characteristic of a single molecular species having a molecular weight of 90,000 daltons. These molecular characteristics are consistent with several reports of the size and shape of activated glucocorticoid receptors from a variety of sources measured in the absence of molybdate (e.g., Hutchens et al., 1981; Norris and Kohler, 1981; Stevens et al., 1979; Wrange et al., 1979), suggesting that the presence of molybdate neither alters the observed hydrodynamic parameters of the activated receptors nor restores to them the parameters characteristic of the nonactivated species. Furthermore, the derived molecular weight (90,000 daltons) of the activated complexes agrees well with the molecular weight of the proposed single polypeptide monomer measured by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis; e.g., Nordeen et al., 1981). In further contrast to the nonactivated receptor complexes, a significant fraction of the activated receptors aggregated into very high molecular weight species that eluted from the S-300 columns at the void volume and sedimented to the bottom of the centrifuge tubes. Thus, receptor activation is consistent with an alteration that promotes, among other changes, the dissociation of the proposed native tetramer into its monomeric subunits. The observed aggregation of a fraction of the activated receptors in the presence of 20 mM molybdate and .15 M KCl further

suggested that the activation process exposes a hydrophobic domain on the receptor surface; this suggestion was strengthened by our further discovery that activation dramatically increased the adsorption of [^{3}H]TA-receptor complexes by glass fiber filters (Whatman GF/C).

It is thus likely that the slowly-sedimenting peak "B" displayed in figure 4-31 represents a partial conversion of the (partially-purified) 77Å complex to activated monomers; the separation of the large complex from associated low molecular weight substances by S-300 chromatography may have increased the concentration of molybdate required to prevent activation during the prolonged sedimentation at moderate ionic strength (e.g., Leach, Grippo, Housley, Dahmer, Salive and Pratt, 1982). The measured size of the activated receptors (58-60 Å) is also in agreement with the radius of the "nonactivated" receptors measured in the absence of molybdate, suggesting that the "nonactivated" receptors had probably become activated during the long incubation without molybdate at 0-4°C and moderate ionic strength.

Our data suggest that perfused mouse brain cytosol contains a single population of putative glucocorticoid receptors and, additionally, a class of CBG-like binders whose source has not been determined. We have found no evidence to support a popular hypothesis (e.g., McEwen et al., 1979; Nestler, Rainbow, McEwen and Greengard, 1981) that neurons contain behaviorally-relevant receptors that preferentially bind corticosterone and cortisol, whereas glial cells may carry receptors having higher affinity for the synthetic agonists TA and dexamethasone.

CHAPTER V

THE BINDING OF CORTICOSTERONE TO A CBG-LIKE COMPONENT OF MOUSE BRAIN CYTOSOL

Introduction

It is often stated that target cell membranes do not present a barrier to free lipophilic steroids, and that their passage from extracellular to intracellular fluid compartments is governed solely by simple diffusion. Recent studies have demonstrated, however, that in at least several different cell types steroid uptake may involve carrier-mediated transport in addition to simple diffusion, and therefore that the ability of a cell to accumulate steroid may be considerably more complex than the simple uptake customarily attributed to the intracellular receptors (for review, see: Giorgi, 1980).

Several studies have established that cortisol and corticosterone are taken up into isolated rat liver cells and purified rat liver plasma membrane vesicles by processes that have the characteristics of carrier-mediated transport (e.g., Rao, Rao, Haller, Breuer, Schattenberg and Stein, 1976; Allera, Rao and Breuer, 1980). The uptake of both steroids was very rapid (cell uptake of cortisol was far more rapid than cortisol binding by liver cytosol receptors), highly temperature-sensitive (reduction of the incubation temperature produced a far greater decrease in the rate of cortisol uptake into cells than in the rate of steroid binding to cytosol receptors in vitro), and was saturable and followed

Michaelis-Menten kinetics. Analysis of initial uptake rates as a function of steroid concentration revealed the presence of both low and high-affinity, saturable uptake systems, in addition to a linear, non-saturable diffusion component. The specificity of the saturable systems was examined by competition experiments using unlabeled steroids; the pattern of results was complex. As expected, corticosterone and cortisol inhibited both systems competitively; dexamethasone inhibited both uptake components noncompetitively, as did both estradiol and testosterone. Biphasic Arrhenius plots relating absolute temperature and the initial rate of cortisol uptake into cells yielded different activation energy barriers for the temperature ranges 5-20°C and 20-32°C, whereas the Arrhenius plot for binding to the cytosol receptors showed a single, lower barrier over the entire temperature range 5-32°C. Abrupt changes in membrane fluidity caused by lipid phase transitions (membrane "freezing" or "thawing") known to occur at temperatures near 10°C (e.g., Inesi, Millman and Eletr, 1973) probably contributed to the dramatic increase in activation energy found below 20°C. Treatment of the cells with metabolic inhibitors abolished both saturable components, leaving intact only the linear diffusion process. Thiol group reagents inhibited the high affinity system, suggesting the involvement of protein sulfhydryl groups in the uptake mechanism. Cell surface carbohydrates were also implicated in the saturable uptake of the steroids into liver cells. There is no obvious explanation for the reported absence of such a saturable, temperature-dependent uptake mechanism in hepatoma (HTC) cells, but it is possible that this membrane transport function may have been lost during the establishment of the

transformed cell line in culture (Rousseau, Baxter, Higgins and Tomkins, 1973).

Another cell type that appears to possess a specific glucocorticoid transport mechanism is the ACTH-secreting mouse pituitary tumor cell AtT-20/D-1, a well-studied glucocorticoid target cell. The saturable uptake of [^{3}H]triamcinolone acetonide (TA) by intact tumor cells was measured at several different temperatures, and the characteristics of this uptake were found to be different from those of steroid binding to receptors in cytosol preparations from these cells (e.g., Harrison, Fairfield and Orth, 1975, 1976; Harrison et al., 1977; Svec and Harrison, 1981). The steroid specificity of the saturable glucocorticoid uptake process was, however, qualitatively similar to that of the isolated cytosol receptors (and therefore quite different from that of plasma CBG, transcortin). When specific initial uptake rates were measured over a wide temperature range a biphasic Arrhenius plot very similar to that reported for liver cells (Rao et al., 1976) was obtained with the pituitary tumor cells; the activation energy for the saturable uptake process changed abruptly at 16°C, again indicating the presence of a lipid phase transition that dramatically decreased the mobility of putative membrane carriers at the lower temperatures. The effects of several potential inhibitors that do not affect the cytosol receptors or alter cell viability were also examined. Neuraminidase and phospholipase A_2 almost completely abolished specific uptake, but they had no effect on the binding of TA to cytosol receptors. Dimethyl sulfoxide (DMSO), ethanol, and the extracellular sulfhydryl inhibitor PCMPS also reduced specific TA uptake dramatically. Thus, the specific uptake mechanism in these cells is sensitive to the loss of sialic acid groups

and to other disruptions of normal membrane organization. A saturable, NAD^+-stimulated glucocorticoid uptake mechanism has also been identified in membrane vesicles prepared from normal human placenta (Fant, Harbison and Harrison, 1979).

While it is not clear how general this saturable uptake phenomenon may be (i.e., in regard to other hormones and target cell types), and while the relative contributions of simple diffusion and specific, carrier-mediated processes to the overall steroid uptake at physiological temperatures are also not known, the experiments reviewed above argue for the involvement of membrane components in specific, saturable glucocorticoid uptake in at least some cell types.

A CBG-like protein has been found in association with both plasma membrane and cytosol fractions from perfused whole pituitaries and from isolated adenohypophysial cells (e.g., Koch et al., 1976; Koch et al., 1981a; Koch, Sakly, Lutz-Bucher and Briaud, 1981b). This CBG-like binder shared many physiochemical characteristics with plasma CBG and also showed the same steroid specificity (i.e., it bound corticosterone but not dexamethasone); furthermore, like CBG it had negligible affinity for nuclear or DNA acceptor sites. The locus of synthesis and the in vivo distribution of the pituitary CBG-like binders are uncertain; they may be cytoplasmic, and a significant concentration of them may also be complexed to the inner or outer plasma membrane surface. Strong evidence has suggested that this macromolecule (when intracellular) can regulate the binding of natural glucocorticoids to the "true" glucocorticoid receptors by competing with receptors for the available intracellular corticosterone, and studies with CBG antisera have suggested that the CBG-like binders may be involved in the uptake of

natural glucocorticoids (but not dexamethasone) into pituitary cells (e.g., de Kloet, Burbach and Mulder, 1977; Koch et al., 1981a,b).

Although the previously discussed saturable glucocorticoid uptake mechanism found in AtT-20/D-1 pituitary tumor cells does not possess the steroid specificity characteristic of CBG, these cells have been found to contain, in addition to the glucocorticoid receptor, an additional glucocorticoid binding site that is present even in cells cultured in serum-free media and that is possibly associated with the plasma membrane; this binding site has much higher affinity for corticosterone than for dexamethasone or TA and may be related to the CBG-like protein found in the normal adenohypophysis (e.g., Harrison, Fairfield and Orth, 1976).

CBG-like proteins have also been found in cytosol fractions prepared from several other tissues, which include uterus (e.g., Al-Khouri and Greenstein, 1980), lung (e.g., Giannopoulos, 1976), liver (e.g., Suyemitsu and Terayama, 1975; Weiser, Do and Feldman, 1979), kidney (e.g., Feldman, Funder and Edelman, 1973; Weiser et al., 1979), heart (Seleznev, Danilov and Smirnov, 1979), skeletal muscle (Mayer, Kaiser, Milholland and Rosen, 1975), and lymphocytes (e.g., Werthamer, Samuels and Amaral, 1973); CBG-like binding sites have also been found associated with liver plasma membranes (Suyemitsu and Terayama, 1975). Immunoreactive CGB-like material has been detected (with highly specific antibodies raised against purified rat plasma CBG) in most liver cells, in many kidney collecting tubule cells, in spleen "white pulp" (containing lymphocytes), some myometrial cells, in scattered anterior pituitary cells, and in most posterior pituitary (but no intermediate lobe) cells from perfused rat tissues (e.g., Siiteri, Murai, Hammond,

Nisker, Raymoure and Kuhn, 1982). These investigators have also reported a large corticosterone-dependent increase in the uptake of [^{125}I]CBG into rat spleen, kidney and uterine tissue. Furthermore, they have observed the dramatic, corticosterone-dependent uptake (and probable nuclear concentration) of FITC-labeled CBG into GH_3 pituitary tumor cells, hepatocytes and lymphocytes (Siiteri et al., 1982).

The observations discussed above have led several groups to suggest that the intracellular or membrane-bound CBG-like binders are derived initially from the circulation (i.e., that they are synthesized in liver), and that they may facilitate the cellular uptake of corticosterone (e.g., Al-Khouri and Greenstein, 1980; Koch et al., 1981; Siiteri et al., 1982). Stimulated by the observation that perfused mouse brain cytosol contains more high-affinity binding sites for corticosterone than for dexamethasone or TA, we have examined the possibility that the mouse brain may also contain a CBG-like binder in addition to the putative glucocorticoid receptor. Furthermore, the experiments have allowed us to further examine the popular hypothesis of glucocorticoid receptor heterogeneity that has been proposed to explain both the different patterns of natural and synthetic steroid uptake by the brain and the different behavioral and neurochemical effects of dexamethasone and corticosterone that have sometimes been observed (e.g., Bohus and de Kloet, 1981; Nestler et al., 1981). We have observed that dexamethasone binds to a subset of the corticosterone binding sites, and that the remaining corticosterone binding sites have many of the characteristics of plasma CBG.

Materials and Methods

Chemicals, Steroids, Isotopes, Animals, Buffers, and Cytosol Preparation

The sources of supplies and the general methods employed have all been described in chapter IV, "Materials and Methods."

Binding Assays

The principal assay used to measure bound [^{3}H]steroid following equilibrium incubations was the DEAE (Whatman DE-81) filter assay, which has been described in chapter IV. Some binding data obtained with the DEAE filter assay were also compared to values obtained with a hydroxyapatite (HAP, Bio Gel HTP) batch assay. This assay was similar to several used previously to measure the binding of steroids to receptor proteins (e.g., Walters and Clark, 1977). For each of the triplicate determinations a 50 l aliquot of the sample was added to 1 ml of a 50% suspension of HAP in buffer A. The samples remained in ice, with occasional vortexing, for 15 min, followed by centrifugation at 2300 x g for 10 min to pellet the HAP. The pellets were then washed 3 times (by resuspension with 2 ml buffer A and recentrifugation) over a 30-45 min period (at 0-4°C), and were then extracted with 2 ml Triton-toluene scintillation cocktail, which was added to scintilation vials with 8 ml additional Triton-toluene fluor for the determination of radioactivity by liquid scintillation. The column and gradient fractions generated by gel charomatography and sedimentation were assayed, following incubation with [^{3}H]steroid, by adsorption of free steroid with dextran-coated charcoal as described below.

Gel Chromatography and Gradient Sedimentation

The Stokes radii (R_s) of unlabeled plasma CBG and the brain cytosol CBG-like binder were determined by gel filtration of samples in buffer A at 0-4°C. Characteristics of the 1.5 x 98 cm Sephacryl S-300 column have been described in chapter IV. A 2 ml aliquot of the unlabeled sample (cytosol, approximately 7.5 mg/ml; plasma, diluted 1:10 in buffer A) was applied to the column, and elution with buffer A was begun. Following elution, specific CBG-like binding was measured in each fraction. Pairs of 165 µl aliquots were removed from each 2 ml fraction and diluted sequentially with 10 µl buffer A containing unlabeled dexamethasone to produce a final concentration of 20nM dexamethasone and then with 75 µl buffer A containing [^{3}H]corticosterone (and, for one of each pair, a 200-fold excess concentration of unlabeled corticosterone) to produce a final [^{3}H]corticosterone concentration of 10nM. Samples were incubated for 4-6 h at 0-4°C, and free steroid was then removed by adsorption to dextran-charcoal. The adsorption assay was performed by adding the 250 µl sample to a 1.5 ml suspension of dextran-coated charcoal (0.75% Norit A activated charcoal, Fisher; 0.075% Dextran T-70, Pharmacia) in buffer A. Following incubation for 5 min at 0-4°C with occasional vortexing, the charcoal was pelleted by centrifugation at 1600 x g for 5 min. The supernatant was then taken for the determination of bound radioactivity by liquid scintillation counting. Calibration of the column with proteins of accurately known Stokes radii has been described in chapter IV.

The sedimentation coefficients of plasma CBG and the brain cytosol CBG-like binder were determined by standard rate sedimentation in glycerol gradients. Samples (250 µl) of unlabeled diluted plasma (1:10)

or brain cytosol (7.5 mg/ml) were centrifuged at 2°C for 25 h at 234,000 x g (average) through 5 ml gradients of 15-35% (w/v) glycerol prepared in buffer A. The cellulose nitrate tubes were punctured, and 175 µl fractions were collected. Pairs of fractions from parallel gradients (which had been loaded with identical samples) were pooled to produce a series of 350 µl samples, and pairs of 165 µl aliquots were taken from each of these pooled samples for the assay of specific CBG-like binding exactly as described above for the determination of specific CBG-like binding in the S-300 column fractions. The value of $S_{20,w}$ for a peak was calculated from the linear regression of $S_{20,w}$ vs. sedimentation distance (Martin and Ames, 1961) for the standard proteins, which were run on parallel gradients in buffer A. The apparent sizes and shapes of the binding sites were determined by standard methods (Siegel and Monty, 1966; Sherman, 1975) as described in chapter IV. The partial specific volume ($\bar{\nu}$) required for the calculations was assumed to be the value reported by Westphal (1975) for human, rat, and rabbit plasma CBG (0.70 cm^3/g).

DEAE and Blue A-Agarose Minicolumn Chromatography

Unlabeled samples of perfused mouse brain cytosol or plasma (diluted 1:40 with buffer A) were chromatographed on Bio-Rad DEAE Bio-Gel A minicolumns (column characteristics: total volume = 5 ml; flow rate = 6 ml/h; fraction volume = 1 ml; maintained at 4°C). The column was washed extensively and equilibrated with buffer A. Samples were run into the column and washed in with 200 µl buffer A. Binding was allowed to proceed (30 min), and the column was then washed with

25 ml buffer A. Bound substances were eluted by a 50 ml linear gradient (0-250 mM KCl in buffer A); fractions (1 ml) were collected. The concentration of specific CBG-like binding in each fraction was assayed exactly as described above for the determination of the Stokes radius by gel chromatography. The elution position of [^{3}H]TA-labeled putative glucocorticoid receptors in perfused brain cytosol was determined by chromatographing samples previously equilibrated with 10nM [^{3}H]TA as described in chapter IV.

The dye Cibacron Blue F-3-GA provides a convenient affinity ligand for serum albumin, the dominant plasma protein. Since this dye does not retain CBG or other α- or β-globulins (e.g., Travis, Bowen, Tewksbury, Johnson and Pannell, 1976), we have enriched CBG and the CBG-like binder in diluted plasma and perfused mouse brain cytosol samples by negative ("drop-through") chromatography on blue A-agarose minicolumns (Amicon). The column (2 ml bed volume) was washed extensively and equilibrated with buffer A. It was then loaded sequentially, at 10 min intervals, with six 0.75 ml aliquots of dilute, unlabeled plasma (diluted 1:40 with buffer A) or unlabeled brain cytosol (2.5 mg protein/ml). The initial plasma or cytosol sample was spiked with a small amount of [^{3}H]water to facilitate complete recovery of the sample volume without massive dilution following its passage through the column. Thus, a 4.5 ml volume of partially-stripped brain cytosol or albumin-stripped plasma was recovered by elution with additional buffer A. The partially-stripped brain cytosol was used to confirm that the blue A-agarose did not retain the CBG-like binder, and the albumin-stripped plasma was used in the attempt to measure the concentration of CBG in mouse plasma.

Determination of the Contamination of Perfused Mouse Brain Cytosol by Residual Blood

Because inulin penetrates the blood-brain barrier only very slowly (e.g., Ferguson and Woodbury, 1969), it may be used to estimate the contamination of brain tissue by residual blood following perfusion. Mice were anesthetized with ether and injected via the tail vein with 3μ Ci [^{14}C]inulin dissolved in 50 μl saline. Two minutes later a blood sample (0.5-1 ml) was taken by heart puncture and perfusion with Hepes-buffered saline (3 ml, pH 7.6) was begun. The duration of perfusion was approximately 5 min. Brains were removed and homogenized, and cytosol samples were prepared in buffer A as described in chapter IV. Plasma samples were prepared by centrifugation. Cytosol and plasma protein concentrations were measured by a modification of the Lowry method (Bailey, 1967), and [^{14}C]inulin radioactivity in triplicate 50 μl aliquots of cytosol and plasma was determined by liquid scintillation counting. These measurements were used, in conjunction with an estimate of the plasma CBG concentration, to estimate the degree of contamination of perfused brain cytosol by blood-borne CBG.

Equilibrium Binding Experimental Designs and Data Analyses

Experimental designs used to measure equilibrium specific binding values (B_{sp}) at different ligand concentrations have been described in chapter IV. When appropriate, ligand affinities and binding site concentrations were determined from Scatchard plots fit to the specific binding data by linear regression; the affinities of unlabeled ligands were determined by the Edsall-Wyman competition experimental design described above in chapters III and IV. Values of specific binding (B_{sp}) were obtained by subtraction of the nonspecific binding (B_{NS}) from

total bound ligand (B_T). Individual experimental designs that have not been described in detail in chapter IV are described below in "Results".

The determination of the affinity of dexamethasone for the receptor subset of the [^{3}H]corticosterone binding sites was determined by generating isotherms with [^{3}H]corticosterone as described in chapter IV, first in the absence and then (using the same cytosol pool) in the presence of a fixed concentration of unlabeled dexamethasone (the Edsall-Wyman design). The isotherm generated without dexamethasone was used to construct a Scatchard plot for the estimation of the total binding site concentration B_{MAX} and a single K_d for the binding of [^{3}H]corticosterone to the complete set of saturable sites. The isotherm generated in the presence of dexamethasone was then used, in conjunction with these estimates of B_{MAX} and K_d and an additional estimate of the total receptor concentration B_o (generated with [^{3}H]dexamethasone for the same pool of cytosol), to calculate the K_{di} for the binding of dexamethasone to a subset of the saturable [^{3}H]corticosterone binding sites. The linear "free-receptor" competition plot (e.g., figure 4-27) that is useful for the determination of K_{di} values within the two ligand-one site context was extended to this very simple special case of the two ligand-two site problem as described below and in chapter III. This extension was facilitated by the absence of a significant dexamethasone-CBG interaction and by the observation that the binding of [^{3}H]corticosterone to the complete set of sites (figure 4-12) was not biphasic. This observation, which agreed with a similar result obtained with rat pituitary cytosol containing both glucocorticoid receptors and CBG-like binding sites (e.g., de Kloet, Wallach and

McEwen, 1975), suggested that the affinity of corticosterone for CBG-like binders was similar to its affinity for the putative glucocorticoid receptors (see also figure 5-2, below, for additional confirmation).

The nomenclature for this special case of the two ligand-two site problem is the following: B_L and B_C are, respectively, the concentrations of specifically-bound [^{3}H]corticosterone and unlabeled dexamethasone (i.e., the competitive inhibitor). B_{LR} and B_{LT} are the concentrations of [^{3}H]corticosterone bound to the putative receptors and to CBG-like binders, respectively: $B_L = B_{LR} + B_{LT}$. B_{MAX} is the total concentration of binding sites, composed of B_o putative glucocorticoid receptors and T_o CBG-like binders: $B_{MAX} = B_o + T_o$. K_d describes the approximately common affinity of [^{3}H]corticosterone for both the receptor and the CBG-like binders, and K_{di} is the equilibrium constant for the binding of dexamethasone to the glucocorticoid receptors. F_L and F_C are the concentrations of free [^{3}H]corticosterone and unlabeled dexamethasone, respectively.

At equilibrium the mass action rate equations for the specific binding of corticosterone and dexamethasone may thus be written as

$$(B_o - B_{LR} - B_C)F_L = K_d B_{LR}, \tag{5-1}$$

$$(B_o - B_{LR} - B_C)F_C = K_{di} B_C, \text{ and} \tag{5-2}$$

$$(T_o - B_{LT})F_L = K_d B_{LT}. \tag{5-3}$$

Equation (5-2) immediately suggests the competition "free receptor" plot (e.g., figure 5-1, inset) described by the equation

$$B_C/F_C = (B_o - B_{LR} - B_C)/K_{di}, \qquad (5\text{-}4)$$

where the bound/free ratio refers to the competing dexamethasone. Equations (5-1) and (5-3) may be combined, using the fact that $B_L = B_{LR} + B_{LT}$, to obtain the expression

$$B_o - B_{LR} - B_C = K_d[(B_L/F_L) - T_o/(F_L + K_d)], \qquad (5\text{-}5)$$

which was used to find the abscissa of the "free receptor" plot. Equation (5-1) may be transformed easily to the expression

$$B_C = B_o - [B_o - B_{LR} - B_C, \text{ free sites}](1 + F_L/K_d), \qquad (5\text{-}6)$$

which was then used, in combination with the identify $F_C = S_C - B_C$, to find the ordinate. The line was fit by simple 2-parameter least-squares linear regression and thus, for statistical reasons, was not constrained to intersect the origin. The value of K_{di} was given by the reciprocal of the slope of the plot.

Results

Equilibrium Binding Parameters for the Mouse Brain Glucocorticoid Binding Sites

The measured values of the equilibrium binding parameters for [^{3}H]corticosterone and [^{3}H]dexamethasone are summarized in table 5-1. Most of these values have been excerpted from table 4-1 and are repeated here in a simplified table for convenience. Individual isotherms for

Table 5-1. Equilibrium binding parameters for [^{3}H]glucocorticoids in perfused mouse brain cytosol. The tabulated parameter estimates are $\bar{x}$ ± s.e.m. Numbers within parentheses indicate either units of measure or the number of independent experiments that have been averaged. Steroid abbreviations are defined in the footnotes to table 4-2.

Parameter	[^{3}H]DEX	[^{3}H]B
B_o [a] (fmole/mg)	370 ± 15(43)	360 ± 60(3)
B_{MAX} [b] (fmole/mg)	---	600 ± 40(15)
K_d [c] ($x10^{-9}$M)	1.8 ± 0.2(18)	7.6 ± 0.6(3)
K_d [d] ($x10^{-9}$M)	---	6.8 ± 0.6(7)
K_{di}[DEX] [e] ($x10^{-9}$M)	---	1.0 ± 0.2(6)
k_{di}[B] ($x10^{-9}$M)	4.7 ± 0.6(4)	---
Cytosol T_o [f] (fmole/mg)	---	230 ± 20(3)
K_d [g] ($x10^{-9}$M)	---	4.7 ± 0.3(3)
Hill "n" [h]	---	0.90 ± .04(3)

a. B_o for putative receptors only; nonspecific correction determined with 200-fold excess DEX for both.

b. Total binding site concentration; nonspecific correction determined with 200-fold excess B.

c. Equilibrium dissociation constants for putative receptors only.

d. Equilibrium constants for complete ensemble of sites.

Continued

Table 5-1 (Continued)

e. Equilibrium constant for the binding of DEX to the receptor subset of the complete ensemble of $[^3H]B$ binding sites.

f. CBG-like binder concentration, measured in the presence of 4 μM DEX to block receptors.

g. Apparent K_d for cytosol CBG-like binder, measured in the presence of 4 μM DEX to block receptors.

h. Hill coefficient for the binding of $[^3H]B$ to the cytosol CBG-like sites.

generated with [^{3}H]dexamethasone, [^{3}H]TA and [^{3}H]corticosterone that are representative of most of the experimental designs have been presented in figures 4-10, 4-11, 4-12, 4-13 and 4-27, which will now be supplemented with the additional figures 5-1 and 5-2.

The isotherms generated with [^{3}H]dexamethasone and [^{3}H]TA were consistent with a single population of high-affinity binding sites (for which [^{3}H]TA has higher affinity than [^{3}H]dexamethasone), and both ligands produced similar estimates of total receptor concentration (B_o). Isotherms describing the binding of [^{3}H]corticosterone to the complete ensemble of saturable sites in brain cytosol were deceptively consistent with a single population of binding sites, but the resulting estimate of total binding site concentration (B_{MAX}) was significantly greater (63% increase) than the estimates obtained with [^{3}H]dexamethasone and [^{3}H]TA. The "merged" or shared affinity of [^{3}H]corticosterone for the complete set of binding sites was lower (K_d was larger) than the receptor affinity measured with [^{3}H]dexamethasone or [^{3}H]TA. Since it seemed likely that this large ensemble of corticosterone binding sites contained a subset pool of contaminating plasma CBG or intracellular CBG-like binding sites that were distinct from the actual glucocorticoid receptors, separate [^{3}H]corticosterone isotherms were also generated by using 200-fold excess unlabeled dexamethasone to saturate only putative glucocorticoid receptors (and not any corticosterone-specific CBG-like binders) in the nonspecific control incubations, thereby removing the contribution of CBG-like binding sites from the derived values of the specific binding (B_{sp}). The isotherms generated in this way were also consistent with a single class of non-cooperative binding sites (i.e., putative receptors), whose estimated concentration was not different

Fig. 5-1. Representative experimental data (generated by the "Edsall-Wyman" experimental design and displayed in the Scatchard coordinate system) used to determine the affinity of dexamethasone for the putative receptor subset of the complete ensemble of [^{3}H]corticosterone binding sites.

The upper isotherm (-DEX, ●) was generated as described in the text and in the legend to figure 4-8, and the lower curve (+3nM DEX, ▲) was generated in an identical fashion with the same pool of cytosol, except that both members of each pair of tubes contained 3 nM unlabeled dexamethasone to affect the "Scatchard" plot as depicted by competition for the glucocorticoid receptor binding sites. The upper line (●) was fit by simple linear regression, and the competition curve (▲) was fit by quadratic polynomial regression; the [^{3}H] corticosterone dissociation constant (K_d) and total binding site concentration (B_{MAX}) were derived from the slope and intercept of the upper [^{3}H]corticosterone Scatchard plot (●).

Inset: representative two-ligand, two-site competition "free receptor" plot used to determine the equilibrium dissociation constant (K_{di}) for the binding of unlabeled dexamethasone to the glucocorticoid receptor subset of [^{3}H]corticosterone binding sites. The points were derived from the data displayed in the Scatchard coordinate system. The nomenclature of the figure conforms to that of chapter III, with the identification of L with [^{3}H]corticosterone and C with dexamethasone. The plot is described in the text and in detail in chapter III. The putative receptor concentration (B_o) was measured by a separate determination with [^{3}H]dexamethasone (not shown), and the abscissa was then calculated by equation (5-5). Equation (5-6) was used, in conjunction with the known dexamethasone concentration (S_C), to find the ordinate, which is the bound/free ratio for the competing dexamethasone. The line (equation 5-4) was fit by simple unconstrained, 2-parameter linear regression. The reciprocal of its slope is the equilibrium dissociation constant (K_{di} or K_{dC}) for dexamethasone. For the specific experiment displayed: K_d = 4.9 nM; B_{MAX} = 4.6 nM (813 fmoles/mg); B_o = 2.6 nM (453 fmoles/mg); the protein concentration was 5.7 mg/ml, and the dexamethasone K_{di} = 0.64 nM (the reciprocal of the slope of the "free receptor" plot).

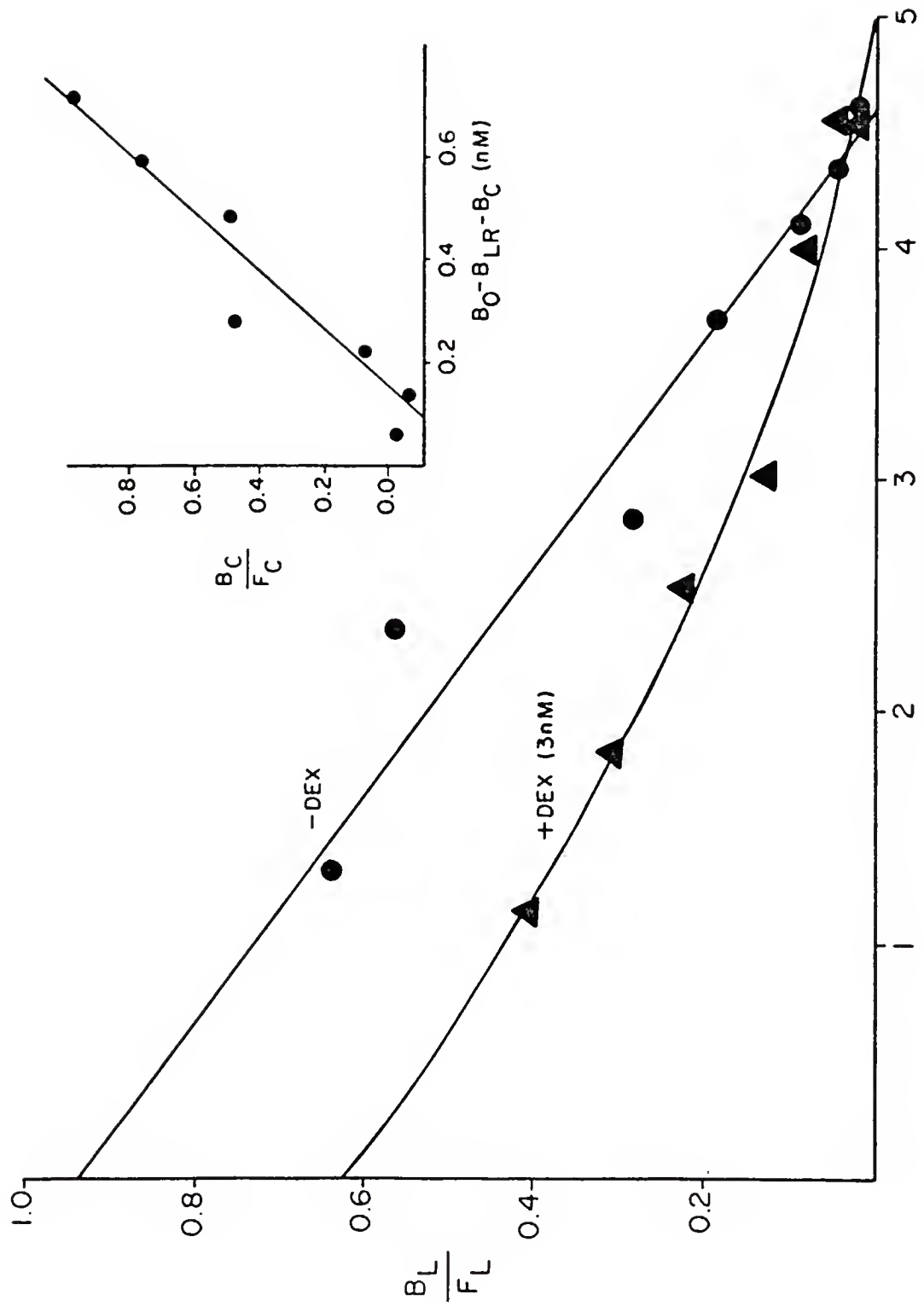
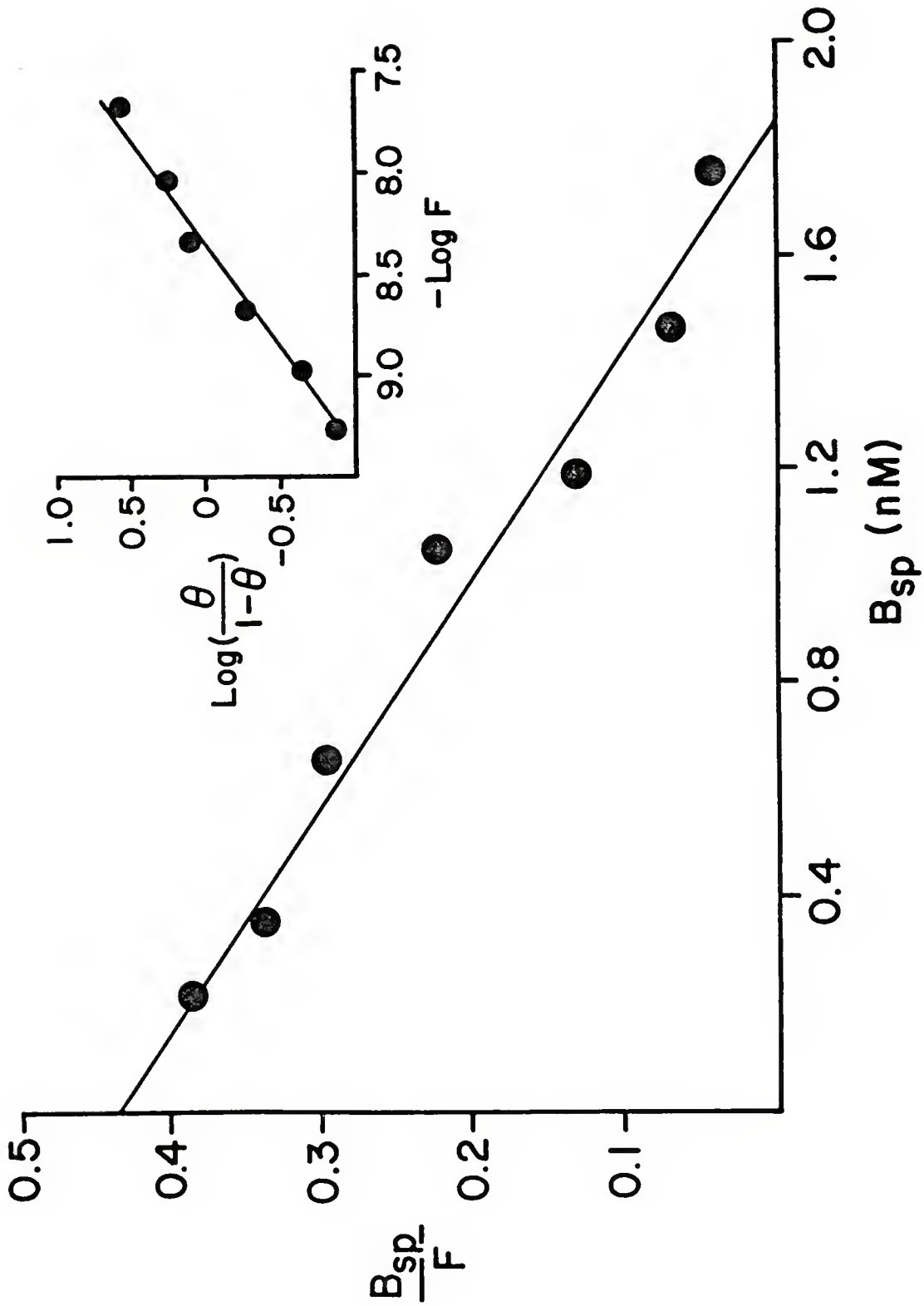

1.0
0.8
0.6
0.4
0.2
$\frac{B_L}{F_L}$
1
2
3
4
5
B_L (nM)
-DEX
+DEX (3nM)
0.8
0.6
0.4
0.2
0.0
$\frac{B_C}{F_C}$
0.2
0.4
0.6
$B_O - B_{LR} - B_C$ (nM)

Fig. 5-2. Scatchard plot depicting the binding of [^{3}H]corticosterone to CBG-like binders in perfused mouse brain cytosol in the presence of 4 μM dexamethasone.

Cytosol was prepared in buffer A, and pairs of samples (250 μl) were incubated for 16 h at 2°C with increasing concentrations of [^{3}H]corticosterone and, for one of each sample pair, a 200-fold excess of unlabeled corticosterone. All incubation tubes also contained 4 μM dexamethasone. Triplicate 50 μl aliquots were then assayed by the DEAE filter method for the determination of total binding (B_T) and nonspecific binding (B_{NS}). Specifically bound steroid (B_{sp}) was determined by subtracting B_{NS} from B_T. The total [^{3}H]corticosterone concentration ranged from 0.8 to 44 nM. The line was fit by least-squares linear regression. Cytosol protein concentration was 7.8 mg/ml. Equilibrium dissociation constant K_d = 4.3 nM, and CBG-like binding site concentration T_o = 1.9 nM (240 fmole/mg cytosol protein). Inset: Hill plot (line fit by linear regression) derived from the same specific binding data combined with the estimate of T_o generated by the Scatchard plot. The regression includes points in the region where binding site occupancy ($\theta = B_{sp}/T_o$) does not exceed .8 (80%). The slope of the line provides the estimated Hill coefficient n = 0.93.


0.5
0.4
0.3
0.2
0.1
$\frac{B_{sp}}{F}$
0.4
0.8
1.2
1.6
2.0
B_{sp} (nM)
1.0
0.5
0
-0.5
$\text{Log}(\frac{\theta}{1-\theta})$
9.0
8.5
8.0
7.5
-Log F

from the estimates obtained with [^{3}H]dexamethasone and [^{3}H]TA. Thus, [^{3}H]corticosterone bound with comparable affinity both to the glucocorticoid receptors and to an additional class of sites that did not recognize the fluorinated agonists.

The affinity of corticosterone for the [^{3}H]dexamethasone binding sites in perfused brain cytosol was measured as described in chapter IV. The value of K_{di}[B] obtained by combining the replications of this experiment (4.7 ± 0.6 nM) was comparable to the value determined by using [^{3}H]corticosterone (± 200-fold excess dexamethasone) to label the receptors directly (7.6 ± 0.6 nM). The discrepancy between these values of K_{di} and K_d for the corticosterone-receptor interaction is probably not unreasonable, since rather different experimental designs were used to generate them (see chapter IV, "Discussion"). The purely competitive displacement of [^{3}H]dexamethasone by corticosterone (figure 4-27) indicated that corticosterone was an endogenous ligand for the putative glucocorticoid receptor labeled by [^{3}H]dexamethasone in brain cytosol.

The affinity of dexamethasone for the receptor subset of the [^{3}H]corticosterone binding sites was also measured. Pairs of cytosol samples were incubated with a series of concentrations of [^{3}H]corticosterone (± 200-fold excess unlabeled corticosterone) as described previously for the construction of an equilibrium isotherm. An additional set of pairs of samples was incubated with the series of concentrations of [^{3}H]corticosterone (± 200-fold excess unlabeled corticosterone) in the presence of a fixed concentration of unlabeled dexamethasone. At the conclusion of the 16 h incubation triplicate samples were taken from each tube for the determination of specific binding (B_{sp} or B_L) with the DEAE filter assay. A representative

experiment is depicted in figure 5-1. The isotherm generated without cold dexamethasone was used to estimate B_{MAX} and the "shared" K_d for the binding of [^{3}H]corticosterone to the receptor and CBG-like subsets of the complete ensemble of saturable sites. The isotherm generated in the presence of cold dexamethasone was used, in conjunction with these estimates of B_{MAX} and K_d, and also an estimate of the receptor concentration B_o generated with [^{3}H]dexamethasone, to compute the K_{di} for the binding of dexamethasone to a subset of the saturable [^{3}H]corticosterone sites as described in chapter III and in "Methods." The high affinity of dexamethasone for this subset of the [^{3}H]corticosterone binding sites (K_{di}[DEX] = 1.0 ± 0.2 nM) was comparable to the receptor affinity derived from Scatchard plots generated with [^{3}H]dexamethasone alone. These data strengthened the evidence that brain cytosol contains two corticosterone binders, a single class of glucocorticoid receptors and a class of CBG-like dexamethasone-resistant binders.

The binding of [^{3}H]corticosterone to the CBG-like binders in perfused brain cytosol was investigated directly by saturating the putative receptors with a high concentration of dexamethasone. Pools of perfused mouse brain cytosol were prepared as described in chapter IV, and pairs of cytosol samples were incubated with a series of concentrations of [^{3}H]corticosterone (± 200-fold excess unlabeled corticosterone). Additionally, all tubes contained 4 μM dexamethasone to saturate fully the glucocorticoid receptors at all concentrations of [^{3}H]corticosterone. Following the 16 h incubation triplicate samples were removed from each tube for the determination of specifically-bound radioactivity by the

DEAE filter assay. A representative isotherm generated in this manner is depicted in figure 5-2. The equilibrium binding parameters generated by these experiments were: total CBG-like binding site concentration T_o = 230 ± 19 (3) fmole/mg protein; K_d = 4.7 ± 0.3 (3) nM; Hill "n" = 0.90 ± .04 (3); the range of protein concentrations for the different experiments was 7.8 - 8.3 mg cytosol protein/ml. The high-affinity CBG-like binder concentration in brain cytosol (T_o) determined by this method agreed precisely with the value determined by subtracting the B_o for [^{3}H]dexamethasone from the B_{MAX} generated with [^{3}H]corticosterone for a given pool of cytosol (230 fmole/mg protein). The apparent affinity of [^{3}H]corticosterone for the CBG-like binders in perfused brain cytosol (determined in the presence of 4 μM dexamethasone) was at least as high as its affinity for the glucocorticoid receptors, suggesting an explanation for the inability of a Scatchard plot depicting the binding of [^{3}H]corticosterone to the complete ensemble of saturable sites to resolve the two classes of binders.

Physicochemical Properties of Mouse Plasma CBG and the Brain Cytosol CBG-like Binders

Representative examples of gel chromatography (Sephacryl S-300) for the determination of the Stokes radii, and rate sedimentation for the measurement of the sedimentation coefficients are depicted in figures 5-3 and 5-4. The sedimentation coefficients, Stokes radii, and the derived molecular weights and shape parameters of the binders from plasma and brain cytosol are listed in table 5-2. The sedimentation coefficients and Stokes radii of the binders from the different sources were identical. The size and shape reported in the table were in reasonable agreement with reported properties of rat, rabbit and human

Fig. 5-3. Comparison of plasma CBG and the CBG-like binder in perfused mouse brain cytosol by gel chromatography on a column of Sephacryl S-300 in buffer A.

Unlabeled cytosol samples (2 ml, approximately 7.5 mg/ml) and diluted plasma (2 ml, diluted 1:10 in buffer A) were chromatographed on a column of Sephacryl S-300 superfine (1.5 x 98 cm) at 12 ml/h in buffer A. Fractions (2 ml) were collected, and the specific CBG-like binding of [^{3}H]corticosterone was assayed in each fraction as described in "Methods". Distribution coefficients (K_d) were calculated from elution volumes (V_e), the void volume (V_o) indicated by blue dextran, and the total liquid volume (V_i) marked by radioactive small molecules ([2-^{3}H]deoxy(D)glucose): $K_d = (V_e - V_o)/(V_i - V_o)$. The column was calibrated with standard proteins, and the Stokes radii of CBG and the CBG-like cytosol binder were calculated from regressions of R_s vs. several functions of the standard protein K_d values as shown in figure 4-30. Means and standard errors from replicates are given in table 5-2.

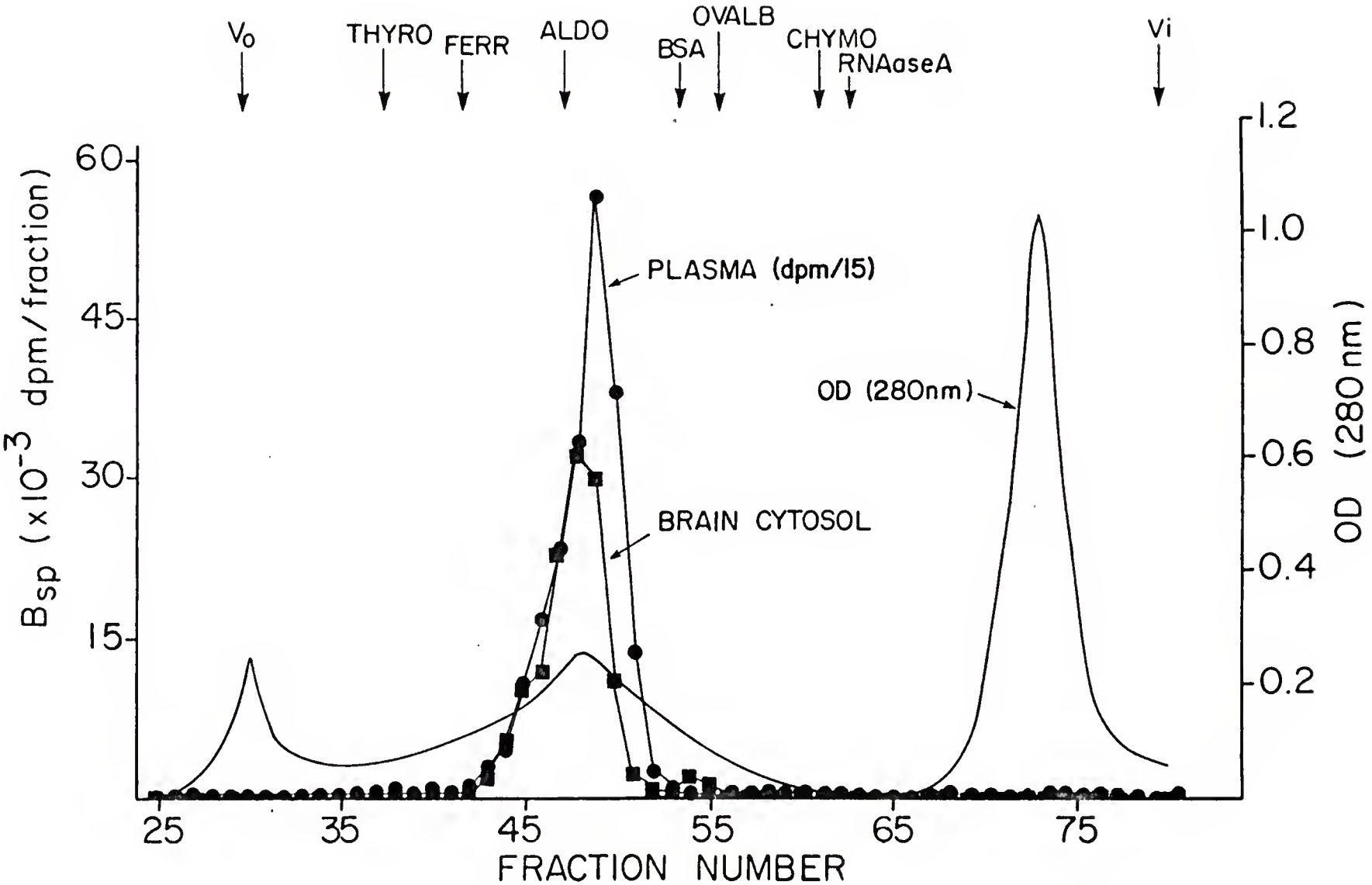

Vo
THYRO
FERR
ALDO
OVALB
BSA
CHYMO
RNAaseA
Vi
PLASMA (dpm/15)
BRAIN CYTOSOL
OD (280nm)
Bsp (x10-3 dpm/fraction)
OD (280 nm)
FRACTION NUMBER
60
45
30
15
1.2
1.0
0.8
0.6
0.4
0.2
25
35
45
55
65
75

Fig. 5-4. Comparison of plasma CBG and the CBG-like binder in perfused mouse brain cytosol by rate sedimentation through glycerol gradients.

Unlabeled cytosol samples (250 µl, approximately 7.5 mg/ml) and diluted plasma (250 µl, diluted 1:10 in buffer A) were centrifuged at $2^{o}C$ for 25 h at 234,000 x g (average) through 5 ml gradients of 15-35% (w/v) glycerol prepared in buffer A. The cellulose nitrate tubes were punctured, and 175 µl fractions were collected. Pairs of fractions from parallel gradients (which had been loaded with identical samples) were pooled to produce a series of 350 µl fractions, which were assayed for the specific CBG-like binding of [^{3}H]corticosterone as described in "Methods". Values of $S_{20,w}$ for the peaks were calculated from the linear regression of $S_{20,w}$ vs. sedimentation distance (Martin and Ames, 1961) for the standard proteins, which were run on parallel gradients as 250 µl samples in buffer A: ovalbumin, 3.6 S; BSA, 4.3 S; IgG, 7.4 S. Standard proteins were [$^{14}CH_3$]-methylated by the method of Rice and Means (1971). Means and standard errors from replicates are presented in table 5-2.

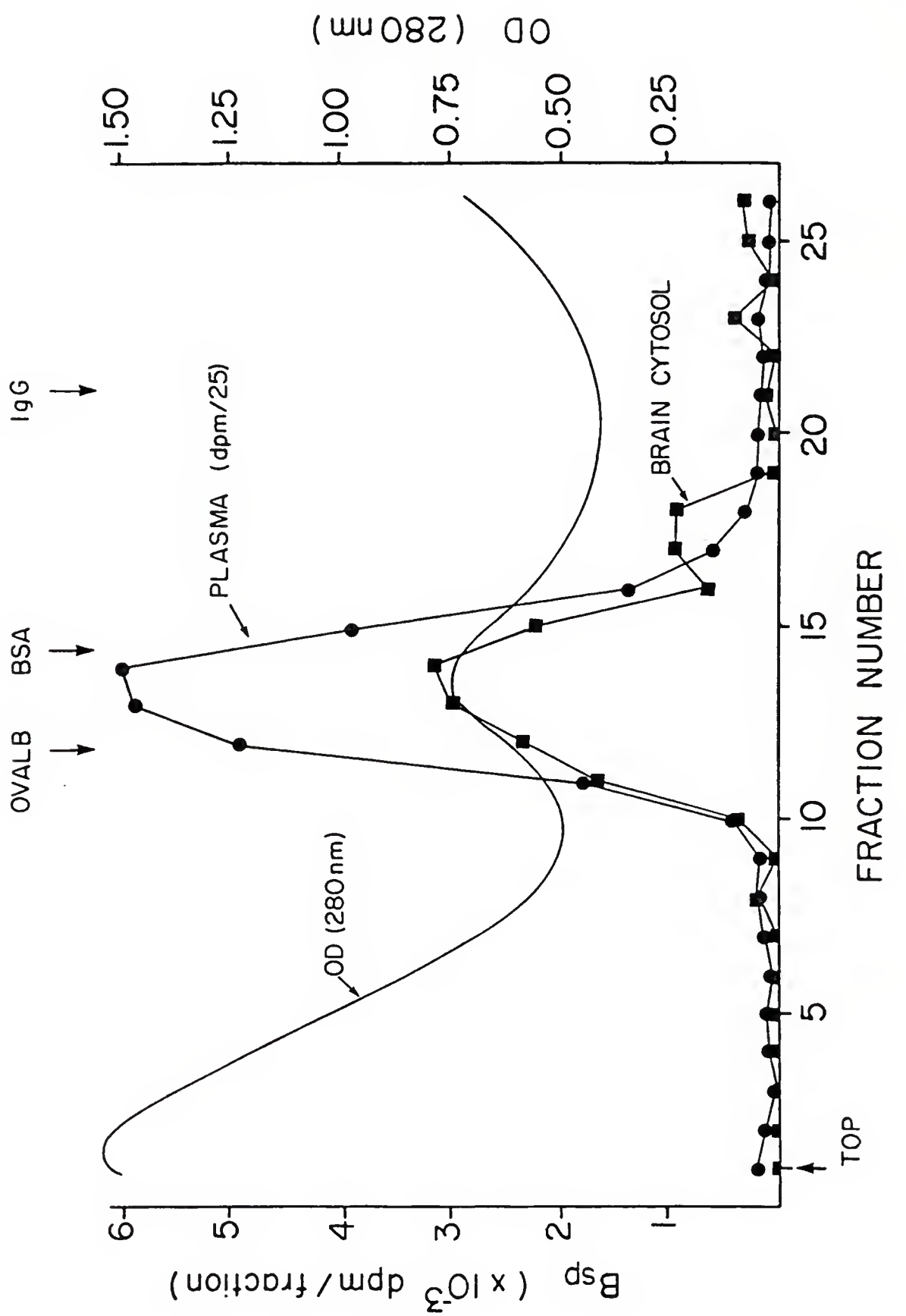

OD (280nm)
1.50
1.25
1.00
0.75
0.50
0.25
IgG
OVALB
BSA
PLASMA (dpm/25)
BRAIN CYTOSOL
OD (280nm)
B_{sp} (x 10^{-3} dpm / fraction)
6
5
4
3
2
1
TOP
5
10
15
20
25
FRACTION NUMBER

Table 5-2. Physical properties of the CBG-like binders in perfused mouse brain cytosol and plasma. Values listed are, where appropriate, $\bar{x} \pm$ s.e.m. The number of experiments is shown in parentheses. See figures 5-3 and 5-4 and "Methods" for details.

Parameter	Brain Cytosol CBG-like Binder	Plasma CBG
R_s ($x10^{-8}$cm)	46 ± 2(3)	46 ± 2(3)
$S_{20,w}$ ($x10^{-13}sec^{-1}$)	4.1 ± 0.1(8)	4.1 ± 0.1(4)
M (daltons)	72,000 ± 5000	70,000 ± 5000
f/f_o (δ=o)	1.70	1.69
b/a (δ=o)	13:1 (prolate)	13:1 (prolate)

plasma CBG (Westphal, 1975). Thus, the size and shape of the cytosol CBG-like binders are very similar to those of plasma CBG. We have been unable to locate published measurements of the size and shape of purified mouse plasma CBG for comparison with the results presented in table 5-2, but a similar sedimentation coefficient (4.25 S) has also been reported for CBG in diluted mouse plasma (Lindenbaum and Chatterton, 1981).

DEAE chromatography of plasma CBG and the brain cytosol CBG-like binder was performed as described in "Methods". In contrast to the more tightly-bound [^{3}H]TA-labeled glucocorticoid receptors (see chapter IV), the CBG and CBG-like binders found in plasma and brain cytosol were eluted from the DEAE Bio-Gel A columns at identical positions near the beginning of the linear salt gradient (0-250nM KCl, not shown), suggesting both that the binders possessed similar surface charge characteristics and that they were only weakly associated with the DEAE Bio-Gel A at the ionic strength of buffer A.

Attempts to Measure the Concentration of Mouse Plasma CBG

The results of our attempts to measure the concentration of plasma CBG in the ovariectomized-adrenalectomized mouse were quite disappointing. Initially, we hoped simply to construct equilibrium isotherms that would reveal the binding affinity of CBG for [^{3}H]corticosterone and the concentration of CBG binding sites in diluted mouse plasma; this estimate of the plasma concentration would then be used to estimate the contribution of blood contamination to the concentration of CBG-like binders in brain cytosol as described below. For this reason pairs of diluted plasma samples (1:25, buffer A) were incubated with a

series of concentrations of [^{3}H]corticosterone (± 200-fold excess unlabeled corticosterone) that ranged from .2 to 75nM. Following the 16 h incubation triplicate samples were removed from each tube for the determination of total (B_T) and specific binding (B_{sp}) by the DEAE filter assay. Scatchard plots constructed with the resulting specific (B_{sp}) and total (B_T) binding data were essentially horizontal for three independent replicates (not shown), suggesting that the diluted plasma contained a vast number of very low affinity binding sites. The "nonspecific" correction was negligible in comparison with the total binding (B_T) at the higher [^{3}H]corticosterone concentrations, suggesting that the observed sites were, however, ultimately saturable. Thus, the concentration of high-affinity CBG in 1:25 diluted plasma could not be measured by this simple method. This outcome was puzzling, since the concentration of CBG in plasma and other complex biological samples has been routinely measured with similar assays (e.g., Rosner, Beers, Awan and Khan, 1976; Siiteri et al., 1982), and since we encountered no problems in the measurement of the concentration of CBG-like binders in brain cytosol by this method (described above).

Although it is potentially dangerous to enrich or partially purify a labile compound during the course of an attempt to measure its concentration in an initial crude preparation, negative ("drop-through") chromatography can sometimes be used to improve an assay without loss of the material of interest. Therefore we removed most of the albumin from diluted plasma (1:40, buffer A) by passing it through small columns of blue F3-GA-agarose as described in "Methods". As a control for the possible loss of CBG, brain cytosol was also passed through blue-A columns; the concentration of CBG-like sites in pools of brain cytosol

was measured as described previously, both before and after negative blue A-agarose chromatography, and no loss of CBG-like material from the cytosol was observed (brain cytosol CBG-like binding site recovery was 109 ± 21%, n=3). A further attempt to measure the concentration of CBG in the diluted, albumin-stripped plasma was then planned. Since it had been reported that the relatively stable CBG molecule was actually labile at higher temperatures (e.g., Siiteri et al., 1982), three pools of diluted plasma and brain cytosol were each divided into four derived samples: untreated, heated (60°C, 30 min), stripped by blue A-agarose, and both stripped by blue A and heated (60°C, 30 min). Each of these derived subpools was split again and incubated with 7.5×10^{-3} M [^{3}H]corticosterone (± 200-fold excess unlabeled corticosterone), and all brain cytosol samples also contained unlabeled dexamethasone to block receptors. Triplicate samples were then removed from each tube for the determination of total (B_T) and specific binding (B_{sp}) by the DEAE filter assay. Again it was impossible to derive a reasonable estimate of the plasma CBG concentration from the data. Although the "nonspecific" corrections were again negligible in comparison with the total plasma binding B_T (indicating ultimate saturability) in all treatment groups, there were no differences among the very large apparent binding site concentrations measured in the four different treatment conditions. Thus, there was no effect of either heat treatment (60°C, 30 min) or albumin removal, and it was not possible to measure the plasma CBG concentration by simply taking the difference between the values of total binding (B_T) measured in the untreated and heat-treated plasma samples. The resulting brain cytosol CBG-like site concentrations were not different from those derived from Scatchard

plots (reported above), and heating had no effect on these cytosol binding site concentrations. Apparently the mouse CBG and CBG-like binding sites were not denatured (in buffer A) by the recommended heat treatment. With the exception of this failure to measure successfully background or "nonspecific" binding by denaturing the high-affinity CBG-like binding sites, difficulties with the assay were limited to the plasma (and not brain cytosol) samples; and we have not yet achieved a satisfactory measurement of mouse plasma CBG concentrations.

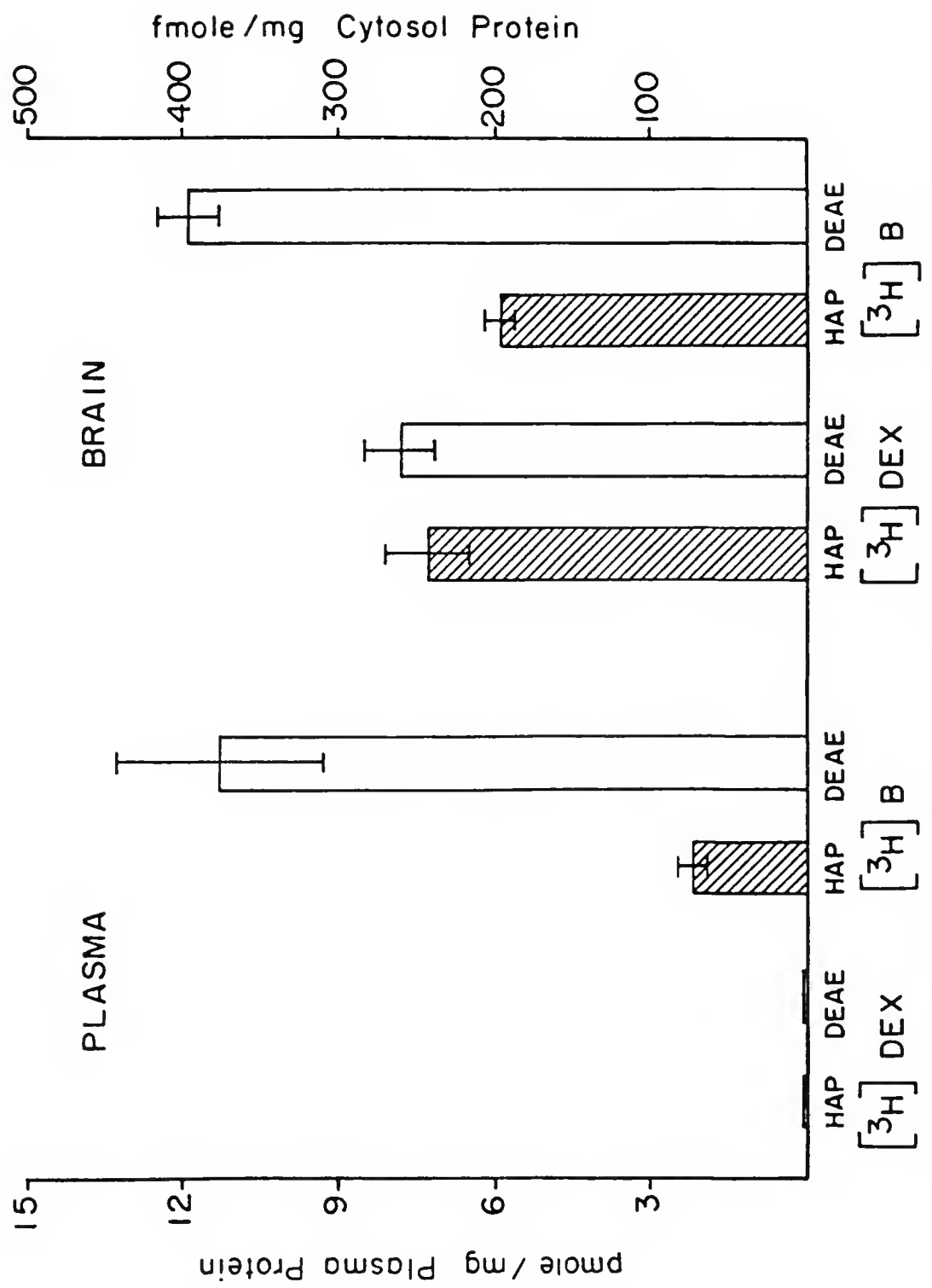
While under the mistaken impression that CBG is completely excluded by hydroxyapatite (e.g., Westphal, 1975), we compared the HAP batch assay and the DEAE filter assay in another single concentration ("saturation") experiment again designed to estimate the total concentration of [^{3}H]dexamethasone and [^{3}H]corticosterone binding sites in both diluted plasma and perfused brain cytosol. Soon after performing this experiment we learned that the binding of CBG to HAP is extremely sensitive to the ionic composition of the buffer, and that it may even vary with the industrial source or individual production batch (e.g., Le Gaillard, Racadot, Racadot-Leroy and Dautrevaux, 1974). We then found that CBG was actually adsorbed quantitatively from plasma diluted with buffer A when it was passed through a column of HAP (Bio-Rad HTP) equilibrated with buffer A (not shown). Thus, our plan to use the HAP batch assay to quantify steroid binding solely to the putative receptors (and not to CBG or CBG-like binding sites) was not possible (without modification of the buffer A formula). We have also subsequently learned that the inclusion of a low concentration of K_2HPO_4 (5 nM) in buffer A does permit the separation of CBG-like sites from receptor sites in this manner, since the receptors remain bound to

HAP until the phosphate concentration has been raised to approximately 100 mM K_2HPO_4 (Luttge and Gray, unpublished). Nevertheless, we now describe the experiment that was performed with HAP equilibrated with buffer A (without added phosphate); the HAP suspension was thus able to adsorb the putative receptors, plasma CBG, and the brain cytosol and diluted plasma samples (1:20, buffer A) were incubated for 16 h at 2°C with 50nM [^{3}H]dexamethasone or [^{3}H]corticosterone (± 200-fold excess homologous unlabeled steroid). Following incubation, triplicate 50 µl aliquots were taken from each sample for the HAP bath assay and also for the DEAE filter assay.

The resulting estimates of total receptor concentration (B_o) measured with [^{3}H]dexamethasone and total binding site concentration (B_{MAX}) measured with [^{3}H]corticosterone are depicted in figure 5-5; the data presented in figure 5-5 were not corrected for assay efficiencies, since we have not yet measured the exact efficiency of the HAP assay. As expected, [^{3}H]dexamethasone failed to bind to plasma proteins as measured by either the HAP assay or the DEAE filter assay. A large difference between the HAP and DEAE assays was observed, however, when [^{3}H]corticosterone was used to label the plasma samples. Initially we believed that this difference reflected the failure of HAP to adsorb CBG. Since we subsequently observed that HAP does adsorb CBG from buffer A, the large difference has now been attributed to the rapid dissociation of [^{3}H]corticosterone from the CBG during the relatively lengthy ($\simeq$ 45 min) washing of the HAP pellets (the half-life $t_{\frac{1}{2}}$ was 30 min for specifically-bound corticosterone in diluted mouse plasma, Lindenbaum and Chatterton, 1981; $t_{\frac{1}{2}}$ was 5 min for purified human

Fig. 5-5. Saturation binding (measured at a single ligand concentration) of [^{3}H]dexamethasone and [^{3}H]corticosterone in diluted blood plasma or perfused mouse brain cytosol, assayed by the hydroxyapatite (HAP) batch assay or by the DEAE filter assay.

Perfused mouse brain cytosol (approximately 4.5 mg/ml) was prepared in buffer A, and plasma samples were diluted 1:20 with buffer A. Pairs of diluted plasma or cytosol samples were incubated for 16 h at 2^{o}C with 50 nM [^{3}H]corticosterone or [^{3}H]dexamethasone and, for one of each sample pair, a 200-fold excess of unlabeled corticosterone or dexamethasone, respectively. Triplicate 50 µl aliquots were then assayed by the DEAE filter method or by use of the HAP batch assay, and the specific binding (B_{sp}) measured by each assay was calculated by subtracting B_{NS} from B_T. No correction for assay efficiency was applied to either the DEAE or the HAP assay results. Range of cytosol protein concentration 4.3 - 4.7 mg protein/ml.


fmole /mg Cytosol Protein
500
400
300
200
100
BRAIN
HAP DEAE
[3H] B
HAP DEAE
[3H] DEX
PLASMA
HAP DEAE
[3H] B
HAP DEAE
[3H] DEX
15
12
9
6
3
pmole /mg Plasma Protein

CBG-cortisol complexes, Rosner, 1972); in contrast, the DEAE assay for achieved a very rapid (1-2 min) separation of bound from free hormone. Since the plasma concentration of free [^{3}H]corticosterone was more than adequate to saturate the high-affinity CBG, an estimate of the plasma CBG concentration was derived from the DEAE filter assay data; this value (15 ± 2.5 pmole/mg plasma protein, n=3, corrected for the DEAE filter assay efficiency) was possibly a considerable overestimate of the actual plasma CBG concentration, since the [^{3}H]corticosterone Scatchard plots constructed with diluted plasma had suggested a vast concentration of low-affinity binding sites. Although we have not yet accurately measured the concentration of plasma CBG, we have used the value listed above in a preliminary calculation (see below) to estimate the contribution of plasma contamination to the observed concentration of brain cytosol CBG-like binding sites.

The concentration of [^{3}H]dexamethasone binding sites measured by both assays was approximately 250 fmole/mg protein (uncorrected for assay efficiency) in perfused brain cytosol. The concentration of [^{3}H]corticosterone binding sites in cytosol was approximately 200 fmole/mg protein when measured with the HAP assay, and it was nearly 400 fmole/mg when assayed by the DEAE filter method. Thus, the binding site concentrations for [^{3}H]dexamethasone and [^{3}H]corticosterone were approximately equal when the contribution of the binding of [^{3}H]corticosterone to CBG-like sites in cytosol was greatly reduced by dissociation during the lengthy HAP assay. The HAP assay measured slightly fewer cytosol [^{3}H]corticosterone binding sites than [^{3}H]dexamethasone binding sites, probably because the dissociation of [^{3}H]corticosterone from the glucocorticoid receptors was much more rapid than the

dissociation of [^{3}H]dexamethasone during the lengthy HAP assay (see chapter VI). Thus, these experimental data had to be completely reinterpreted following the discovery that CBG was adsorbed by HAP from buffer A.

Estimation of the Contribution of Blood Contamination to the Concentration of CBG-like Binding Sites in Brain Cytosol

The data from the [^{14}C]inulin washout experiment were used, in conjunction with the estimate of plasma CBG concentration listed above, to estimate the degree of contamination of perfused brain cytosol by blood-borne CBG. The contamination was estimated by the following calculation:

$$C = (A \times B \times E)/(D \times F), \qquad (5\text{-}7)$$

where C = contamination of cytosol by plasma (fmoles blood-borne CGB-like sites/mg brain cytosol), A = plasma CBG concentration (fmoles CBG/mg plasma protein), B = plasma protein concentration (mg/ml), D = plasma inulin concentration (dpm [^{14}C]inulin/ml plasma), E = cytosol inulin concentration (dpm [^{14}C]inulin/ml cytosol), and F = cytosol protein concentration (mg/ml). The assumed value of plasma CBG concentration (A = 15,000 fmoles/mg protein) was taken from the DEAE filter assay data (corrected for assay efficiency) presented in figure 5-5. The calculation also assumed that the washout of CBG and of inulin from the brain circulation during perfusion were equivalent.

The resulting estimate of the contamination was C = 92 ± 13 (fmole plasma CBG sites/mg brain cytosol protein, n = 5); for the reasons described above a large uncertainty (not reflected in the s.e.m.

listed above) was associated with this estimate, which we must refine as soon as problems connected with the plasma CBG assay have been solved. The estimated value of the contamination (C = 92 fmole/mg) suggested that the contribution of blood-borne CBG to the concentration of CBG-like binding sites in cytosol was approximately 40% of the total concentration of CBG-like sites measured in the perfused brain cytosol (T_o = 230 fmoles/mg protein). Because of the large uncertainty in the estimated plasma CBG concentration, the inulin data were inconclusive, and it was not possible to reject the possibility that all cytosol CBG-like sites resulted from incomplete perfusion. (It is also possible, of course, that the contamination was significantly less than 40%.)

Discussion

Perfused mouse brain cytosol appeared to contain only a single class of putative glucocorticoid receptors to which both corticosterone and the fluorinated agonists bound with high affinity; the additional dexamethasone-resistant corticosterone binding sites were closely related to plasma CBG, having indistinguishable size, shape, and surface charge characteristics that were quite distinct from those of the receptors. Furthermore, the brain cytosol CBG-like binding sites possessed the high affinity (in the nanomolor range) that is characteristic of plasma CBG (e.g., Westphal, 1975; Lindenbaum and Chatterton, 1981). Thus, we have found no evidence for the suggestion of some investigators (e.g., McEwen et al, 1979; Nestler et al., 1981) that neurons contain receptors that preferentially bind corticosterone and cortisol, whereas glial cells possess receptors having higher affinity for the synthetic agonists.

The source and compartmentation of the mouse brain CBG-like binders in vivo remain uncertain. The inulin washout data indicated the presence of some blood contamination in the cytosol, but the large uncertainty associated with the estimated plasma CBG concentration made it impossible to determine the exact contribution of this contamination to the observed concentration of cytosol CBG-like binding sites. Efforts to obtain a precise measurement of the plasma CBG concentration have now been planned. Since it is possible that the problems associated with this assay may have resulted from an excessive molar concentration of binding sites in the plasma, we shall repeat the measurements using very dilute plasma samples (diluted in the range 1:250 to 1:1000; e.g.; Siiteri et al., 1982; Lindenbaum and Chatterton, 1981). If necessary, the plasma CBG will also again be partially purified manyfold prior to the assay by tandem negative chromatography through blue A-agarose (as described above) followed by passage through hydroxyapatite (HAP) in the presence of 5-10 mM K_2HPO_4. Furthermore, a better correction for nonspecific binding will be obtained following the denaturation of plasma CBG binding sites in control samples by exposure to a higher temperature (e.g., 83°C for 30 min; Svec and Harrison, 1981). Although the extent of the contamination of cytosol by plasma is uncertain, it remains possible that much of the brain CBG-like binder is either intracellular or bound to the external plasma membranes. Additional studies of the effects of systematic injections of antibodies to mouse CBG on the concentration of brain cytosol CBG-like sites and studies of the potential steroid-dependent brain uptake of [^{125}I]CBG obviously are required. A strong correlation between the concentration of pituitary cytosol CBG-like

binding sites and the level of circulating CBG has been observed following experimental manipulations (e.g., thyroidectomy, adrenalectomy, treatment with thyroid hormones) that altered the plasma CBG concentration (e.g., Koch et al., 1981b); it will be interesting to learn if the concentration of mouse brain cytosol CBG-like sites is also correlated with the circulating CBG concentration.

The reported studies of rat brain cytosol glucocorticoid binding sites have yielded conflicting results concerning the existence of intracellular CBG-like sites. Equal concentrations of binding sites for [^{3}H]dexamethasone and [^{3}H]corticosterone were found in perfused rat hippocampal cytosol (de Kloet et al., 1975), and dexamethasone completely displaced the specific binding of [^{3}H]corticosterone in the same preparation (Wrange, 1979). In contrast, low concentrations of CBG-like material have also been observed in rat hippocampal cytosol (MacLusky et al., 1977; Meyer, Leveille, de Vellis, Gerlach and McEwen, 1982).

Although there is no evidence for a saturable, carrier-mediated, transmembrane steroid transport mechanism composed of a limited number of membrane-embedded CBG-like molecules, the possible role of CBG as a promoter of rapid steroid uptake has been suggested (e.g., Koch et al., 1981a; Siiteri et al., 1982). Such a role for CBG could also contribute to the more rapid uptake of [^{3}H]corticosterone than [^{3}H]dexamethasone that has been observed in many experiments (e.g., de Kloet et al., 1975). Intracellular CBG might modulate the hormonal response system by partially shielding the receptors, decreasing the apparent intracellular (free) steroid concentration. This would not alter the maximal response of the system, but different amounts of intracellular CBG could allow

different tissues to develop their typical responses over somewhat different ranges of circulating steroid concentration.

CHAPTER VI

KINETICS OF MOUSE BRAIN GLUCOCORTICOID RECEPTOR ASSOCIATION AND DISSOCIATION

Introduction

Studies of glucocorticoid binding kinetics have consistently shown a lack of agreement between equilibrium and kinetic estimates of the equilibrium constants (e.g., Pratt et al., 1975; Yeakley et al., 1980); the dissociation constant K_d calculated as the ratio of the rate constants has been smaller (indicating higher affinity) than that obtained from equilibrium isotherms. Typically, the discrepancies have been highly correlated with the affinity of the ligand and are largest for the synthetic high-affinity agonists (TA and dexamethasone), but even the physiological steroid [^{3}H]corticosterone has usually produced a significant (several-fold) discrepancy. For example, discrepancies of 79-fold, 12-fold, and 7-fold were reported for measurements with [^{3}H]TA, [^{3}H]dexamethasone, and [^{3}H]corticosterone, respectively, in pituitary tumor (AtT-20) cell cytosol (Yeakley et al., 1980). The simplest explanation of this discrepancy is that the duration of the equilibrium incubations was not adequate, particularly for the higher-affinity ligands that require longer incubations for the attainment of equilibrium; inadequate incubation always leads to an overestimate of the equilibrium k_d. Another explanation is that in the equilibrium studies unbound receptors were being "decapacitated" (losing functional binding sites) more rapidly than bound receptors, and thus that equilibrium was

never attained, even with prolonged incubations. Another possible, if unlikely, explanation is that in the kinetic experiments the forward rate constant was overestimated because the total receptor concentration B_o (present at the beginning of the association reaction) was underestimated; this can happen in a kinetic study if the receptor concentration B_o is determined after maximal binding has been attained, many hours after the beginning of association, at a time when some receptor loss has occurred. It was therefore of interest to see if this discrepancy between the independently determined values of K_d could be minimized by reducing or eliminating the inactivation of unoccupied receptor sites in vitro during the necessarily lengthy equilibrium incubations by the inclusion of molybdate and other protective ingredients in the receptor buffer A, since unoccupied sites are not lost at a significant rate in buffer A (see chapter IV). Furthermore, since molybdate prevents the activation of receptors to the nuclear binding form (chapter IV), both kinetic and equilibrium experiments may now be performed with a single population of nonactivated receptors; thus, the inclusion of molybdate should prevent any complexities or discrepancies that might result from the possible possession by nonactivated and activated receptors of different dissociation rate constants.

In order to compare equilibrium constants derived independently from equilibrium and kinetic experiments, we have therefore measured the association and dissociation rate constants describing the interactions of [^{3}H]corticosterone, [^{3}H]dexamethasone, and [^{3}H]TA with mouse brain cytosol glucocorticoid receptors. The second order rate constant of association was determined for each steroid by measuring the amount of [^{3}H]steroid specifically bound following the incubation of cytosol

samples with a fixed concentration of [^{3}H]steroid for different short durations, and the first order dissociation rate constant was determined by simply measuring the amount of [^{3}H]steroid remaining specifically bound at different times following perturbation of an equilibrium incubation by the sudden removal of free [^{3}H]steroid or by dilution of the specific activity of the free [^{3}H]steroid by a large factor, or by both. The resulting estimates of the association and dissociation rate constants have also been used to generate predictions, for various combinations of steroid and receptor concentrations, of the times required for steroid-receptor interactions to attain equilibrium (see chapters II and IV).

Large concentrations of progesterone and other glucocorticoid antagonists and partial agonists (but not optimal glucocorticoid inducers such as dexamethasone and aldosterone) significantly and reversibly increased the dissociation rate of previously-formed glucocorticoid-receptor complexes both in vivo (pituitary AtT-20 tumor cells) and in rat liver, kidney, thymus, pituitary, pituitary tumor cell (AtT-20), uterine, and hippocampal cytosol preparations; these data have suggested the existence of a second, lower-affinity, allosteric binding site for glucocorticoid antagonists, the occupancy of which lowers the steroid-binding affinity of the primary glucocorticoid binding site by accelerating the dissociation of the glucocorticoid-receptor complexes (Suthers, Pressley and Funder, 1976; Jones and Bell, 1980; Svec, Yeakley and Harrison, 1980; Moguilewsky and Deraedt, 1981).

Although the dissociation effect was graded and saturable, the steroid concentrations required to accelerate the dissociation of [^{3}H]glucocorticoids were enormous and nonphysiological, when the

steroids were added after, rather than before, occupation of the glucocorticoid receptors by [^{3}H]dexamethasone; when experiments were performed in this way the values of K_m for the acceleration of glucocorticoid dissociation (80 μM for progesterone and 20 μM for the synthetic progestin R-5020) were 1000-fold greater than the concentrations of antiglucocorticoids required to competitively inhibit the binding of [^{3}H]dexamethasone to the primary binding sites (Svec et al., 1980). However, when receptors were preincubated with progestins before the binding of [^{3}H]dexamethasone, the progestins accelerated the dissociation of glucocorticoid-receptor complexes at 500-fold lower concentrations that were in the range required for physiological antiglucocorticoid activity and the competitive inhibition of [^{3}H]glucocorticoid binding. Furthermore, the dissociation of [^{3}H]progestins from their own uterine and pituitary cytosol receptors was accelerated by preincubation with glucocorticoids (Moguilewsky and Deraedt, 1981). Although the situation is extremely complex, these results suggest a reciprocal inhibitory relationship between glucocorticoids and progestins that is mediated by actions at secondary, allosteric binding sites. We have investigated the accelerated dissociation of mouse brain cytosol [^{3}H]dexamethasone-receptor complexes produced by the addition of high concentrations of progesterone.

Materials and Methods

Supplies and Cytosol Preparation

The sources of supplies and the general methods employed, including the DEAE filter assay, have all been described in chapter IV, "Materials and Methods".

Kinetic Experimental Designs and Data Analyses

The association rate constant (k_a) was determined by measuring specific binding (B_{sp}) with the rapid DEAE filter assay as a function of time (t) for a relatively short period (during which dissociation could be neglected) following the mixing of ligand with the receptor preparation. If t is small in comparison with the half-life of the bound complex ($t_{\frac{1}{2}}$), then dissociation may be neglected and the rate equation (2-1) becomes simply

$$dB_{sp}/dt = k_a(S_L-B_{sp})(B_o-B_{sp}), \qquad (6\text{-}1)$$

where S_L is the total [^{3}H]ligand concentration and B_o is the total receptor concentration. Upon integration with the obvious initial condition that $B_{sp} = 0$, equation (6-1) yields

$$k_a t(S_L-B_o) = \ln(B_o/S_L) + \ln[(S_L-B_{sp})/(B_o-B_{sp})]. \qquad (6\text{-}2)$$

Thus, at short times a plot of $\ln[(S_L-B_{sp})/(B_o-B_{sp})]$ as a function of time is a line with slope $k_a(S_L-B_o)$, from which the association constant k_a is derived immediately. The estimate of the functional receptor concentration (B_o) required for the calculation was derived from an equilibrium experiment performed as described in chapter IV with the same cytosol preparation used for the kinetic experiment.

For each determination a pair of cytosol samples was prepared in buffer A, and initially (t=0) the cytosol samples (1.125 ml) were mixed with 0.375 ml buffer A containing sufficient [^{3}H]glucocorticoid (and, for one sample of the pair, a 200-fold excess unlabeled homologous

steroid or, for the case of [^{3}H]corticosterone, a 200-fold excess unlabeled dexamethasone) to produce the desired final [^{3}H]ligand concentration (in the range 10-30 nM). Triplicate 50 µl aliquots were removed from each sample of the pair as rapidly as possible (approximately every 7 min) for the determination of specifically-bound [^{3}H]ligand (B_{sp}) by the DEAE filter assay. A plot of ln [(free steroid)/(free receptor)] versus time was constructed as described (incorporating the independently-determined estimate of B_o); and the best-fitting line, whose slope provided the estimate of k_a, was determined by least-squares linear regression. For the several association experiments with [^{3}H]dexamethasone the protein concentration range was 7.3-10.3 mg protein/ml, and the range of the total [^{3}H]dexamethasone concentration (S_L) was 9.9 - 24 nM; the range of the receptor concentration (B_o) was 2.9 - 4.0 nM (248 - 388 fmole/mg cytosol protein). For the [^{3}H]TA association experiment the cytosol protein concentration was 11.3 mg/ml, and the concentration of [^{3}H]TA was 17.4 nM; the receptor concentration (B_o) was 4.4 nM (390 fmole/mg protein). For the different [^{3}H]corticosterone association experiments the protein concentration range was 3.1 - 4.3 mg/ml, and the range of the total [^{3}H]corticosterone concentration (S_L) was 18.5 - 27 nM; the range of the receptor concentration (B_o) was 1.1 - 1.8 nM (265-468 fmole/mg protein).

The dissociation rate constant k_d was determined by measuring the remaining specifically-bound ligand (B_{sp}) as a function of the time following some manipulation designed to prevent further association (or reassociation) of labeled ligand with the receptors. These manipulations included adsorption of the free labeled steroid onto dextran-charcoal, followed by dilution of the preparation to further

reduce the concentration of free labeled steroid to a negligible level, and the addition of high concentrations of unlabeled steroids to block the further association of [^{3}H]steroid with the receptors; in some experiments high concentrations of unlabeled progesterone were added both to accelerate the dissociation of the remaining [^{3}H]dexamethasone-receptor complexes and to block further association of [^{3}H]dexamethasone with the glucocorticoid binding sites. Thus, when the association of [^{3}H]steroid is blocked, the rate equation (2-1) becomes

$$dB_{sp}/dt = -k_d B_{sp} \qquad (6\text{-}3)$$

Integration yields the familiar first order dissociation relations

$$\ln[B_{sp}/B(0)] = -k_d t \qquad (6\text{-}4)$$

and finally

$$B_{sp}(t) = B(0)\exp(-k_d t), \qquad (6\text{-}5)$$

where B(0) is the initial concentration of specifically-bound ligand at the beginning of the dissociation period. The rate constant k_d is the slope of the plot of $\ln[B_{sp}(t)/B(0)]$ as a function of the dissociation time t. The dissociation rate is frequently also reported as the half-life ($t_{\frac{1}{2}}$) of the bound complex:

$$t_{\frac{1}{2}} = (\ln 2)/k_d \; . \qquad (6\text{-}6)$$

The first order rate constant describing the dissociation of [^{3}H]dexamethasone from the brain cytosol glucocorticoid receptors at 0-2°C was measured under conditions of both decreasing and constant receptor occupancy, and also in the presence of several different high concentrations of progesterone (spaced equally on a log scale). A pair of cytosol samples (designated X and Y) was prepared in buffer A as described in chapter IV; these pools of cytosol were then incubated to saturation at 2°C for 12 h with 6 x 10^{-8}M [^{3}H]dexamethasone and, for sample Y of the pair, 200-fold excess unlabeled dexamethasone. The free dexamethasone was then removed from each sample by three sequential treatments with dextran-coated charcoal (DCC). For each DCC adsorption the sample was added to a pellet produced by centrifugation of an equal volume of dextran-charcoal suspension (0.5% Norit A, 0.05% Dextran T-70) at 1600 x g for 5 min. Following resuspension of the charcoal pellet and incubation at 0-2°C for 5 min, the DCC was again pelleted by centrifugation at 1600 x g for 5 min and the cytosol supernatant was removed. Following the third sequential DCC adsorption the cytosol pools (X and Y) were used to monitor the dissociation of [^{3}H]dexamethasone-receptor complexes under several different conditions.

A series of flasks containing 75 ml buffer A and either no additional steroid (flask X_0), 25 μM dexamethasone (flask X_1), or one of several different concentrations of progesterone (flasks X_i) was prepared; 1.5 ml aliquots were then removed from the labeled, DCC-treated cytosol pool X and mixed with the contents of these flasks. Another 1.5 ml aliquot was removed from pool X and added to a flask prepared with 75 ml buffer A containing 6 x 10^{-8}M [^{3}H]dexamethasone (flask X_t). A 1.5 ml aliquot of the DCC-treated cytosol pool Y was then

diluted into a flask (Y_0) prepared with 75 ml buffer A containing 25 μM unlabeled dexamethasone, and another 1.5 ml aliquot was added to a flask (Y_1) prepared with buffer A containing 6 x 10^{-8}M [^{3}H]dexamethasone and 200-fold excess cold dexamethasone. The flasks were incubated in ice in a cold room at 0-2°C. Triplicate 2.5 ml samples were removed periodically from each flask for the determination, by additional dextran-charcoal assays, of the remaining specifically-bound [^{3}H]dexamethasone in each of the flasks X_0, X_1, and each of the X_i flasks. Each of these values of $B_{sp}(t)$ was determined by subtracting the nonspecific background correction determined simultaneously by the assay of samples from the Y_0 flask. The (unchanging) concentration of specifically-bound [^{3}H]dexamethasone in flask X_t (whose nonspecific correction was determined by the assay of samples from flask Y_1) served as a control to detect any loss of functional binding sites in vitro during the course of the dissociation experiment. The 2.5 ml aliquots of diluted cytosol from the flasks were assayed by adding them to pellets produced by centrifugation of equal volumes of the dextran-charcoal suspension (0.5% Norit A, 0.05% dextran T-70) at 1600 x g for 5 min. Following resuspension of the DCC pellets and further incubation at 0-2°C for 5 min, the charcoal was again pelleted by sedimentation at 1600 x g and the cytosol supernatants removed for scintillation counting in the Triton-toluene cocktail described in chapter IV. The resulting values of $B_{sp}(t)$ were then used to calculate, as described above, the dissociation constants (k_d) for the different conditions. In a single experiment the rate constant describing the dissociation of [^{3}H]TA from the cytosol glucocorticoid receptors at 0-2°C in buffer A was determined at high occupancy (in the

presence of 25 μM unlabeled TA) exactly as described above for [^{3}H]dexamethasone.

The rate constant describing the dissociation of [^{3}H]corticosterone from the brain cytosol glucocorticoid receptors at 0-2°C in buffer A was measured at high receptor occupancy. A pair of cytosol samples (designated C and D) was prepared in buffer A as described in chapter IV; these pools of cytosol were then incubated at 2°C for 12 h with 6×10^{-8}M [^{3}H]corticosterone and, for sample D of the pair, 200-fold excess unlabeled dexamethasone to saturate the putative receptors but not the dexamethasone-resistant CBG-like binding sites. Following the incubation, two 1.125ml aliquots (samples C_1 and C_2) were removed from cytosol pool C; to C_1 was added 0.375ml buffer A containing sufficient cold dexamethasone to produce a concentration of 25 μM in the final 1.5 ml sample, and to C_2 was added 0.375 ml buffer A alone. To a 1.125 ml aliquot of sample D was added 0.375 ml buffer A containing cold dexamethasone to produce a concentration of 25 μM in the final 1.5 ml sample (designated D_1), and all tubes were returned to ice in a cold room. Triplicate 50 μl aliquots were removed periodically from each sample for the determination, by the DEAE filter assay, of the remaining specifically-bound [^{3}H]corticosterone in sample C_1. Each value of $B_{sp}(t)$ was determined by subtracting the nonspecific background measured simultaneously by the assay of sample D_1. The (unchanging) concentration of specifically-bound [^{3}H]corticosterone in sample C_2 (whose nonspecific correction was also determined by the aliquots from sample D_1) served as control for the potential loss of functional receptors in vitro during the course of [^{3}H]corticosterone dissociation.

The values of $B_{sp}(t)$ from sample C_1 were then used to calculate, as described above, the dissociation rate constant k_d.

Results

Steroid Association Rates

Figures 6-1, 6-2, and 6-3 depict representative experimental determinations of the rate constants (k_a) for the association of [^{3}H]dexamethasone, [^{3}H]TA, and [^{3}H]corticosterone, respectively, with putative glucocorticoid receptors in perfused mouse brain cytosol; summary results with standard errors for the association rate constants measured with the three [^{3}H]ligands are presented in table 6-1. The rate constants were determined by measuring, for each replication of the experiment, the concentration of steroid-receptor complexes as a function of the incubation time for a given total ligand concentration. The association rate constants were 6.9×10^5 M^{-1} min^{-1} for dexamethasone, 4.4×10^5 $M^{-1}min^{-1}$ for TA, and 4.5×10^5 $M^{-1}min^{-1}$ for corticosterone. These rate constants were very similar to each other, and they were within the range of the previously-reported values for rate constants describing the association of these ligands with glucocorticoids receptors in other tissues (e.g., Kaine, Nielsen and Pratt, 1975; Yeakley et al., 1980; Aranyi, 1982).

It is also possible to determine the association constant k_a by measuring specific binding following brief incubations of constant, short duration conducted at different ligand concentrations. These values of B_{sp}, when divided by the incubation time, are taken as estimates of the initial rate of increase of binding (dB_{sp}/dt), which is plotted on the ordinate as a function of the total ligand S_L. The

Figure 6-1. Representative experimental determination of the second-order rate constant describing the association of [^{3}H]dexamethasone with glucocorticoid receptors in perfused mouse brain cytosol at 0-2°C.

A pair of cytosol samples was prepared in buffer A, and at time zero (t=0) the samples were mixed with small volumes of buffer A containing sufficient [^{3}H]dexamethasone (and, for one sample of the pair, a 200-fold excess of unlabeled dexamethasone) to bring the final concentration of [^{3}H]dexamethasone to 18.5 nM. Triplicate 50 µl aliquots were removed from each sample of the pair as rapidly as possible (approximately every 7 min) for the determination of specifically-bound [^{3}H]dexamethasone (B_{sp}) by the DEAE filter assay. The receptor concentration (B_o) required for the calculation of the ordinate was derived (as described in chapter IV) from an equilibrium experiment performed with the same cytosol preparation (not shown; figure 4-10 is a representative example). The abscissa of the plot is the time elapsed from the initial mixing of [^{3}H]dexamethasone with the receptor preparation, and the ordinate is ln[(free ligand)/(free receptor)]. For short times the plot of this ordinate as a function of time is a line having slope $k_a(S_L - B_o)$, from which the association rate constant k_a may be derived immediately. The line was fit by least-squares linear regression. Association rate constant $k_a = 6.84 \times 10^5 M^{-1} min^{-1}$; receptor concentration B_o = 2.3 nM (295 fmole/mg cytosol protein); total [^{3}H]dexamethasone concentration was 7.8 mg/ml.
Inset: data describing the appearance of specifically-bound [^{3}H]dexamethasone (B_{sp}) as a function of the time after mixing the cytosol with [^{3}H]dexamethasone. The data were transformed as described above and used, in conjunction with the estimate of receptor concentration (B_o), to calculate the association rate constant (k_a).

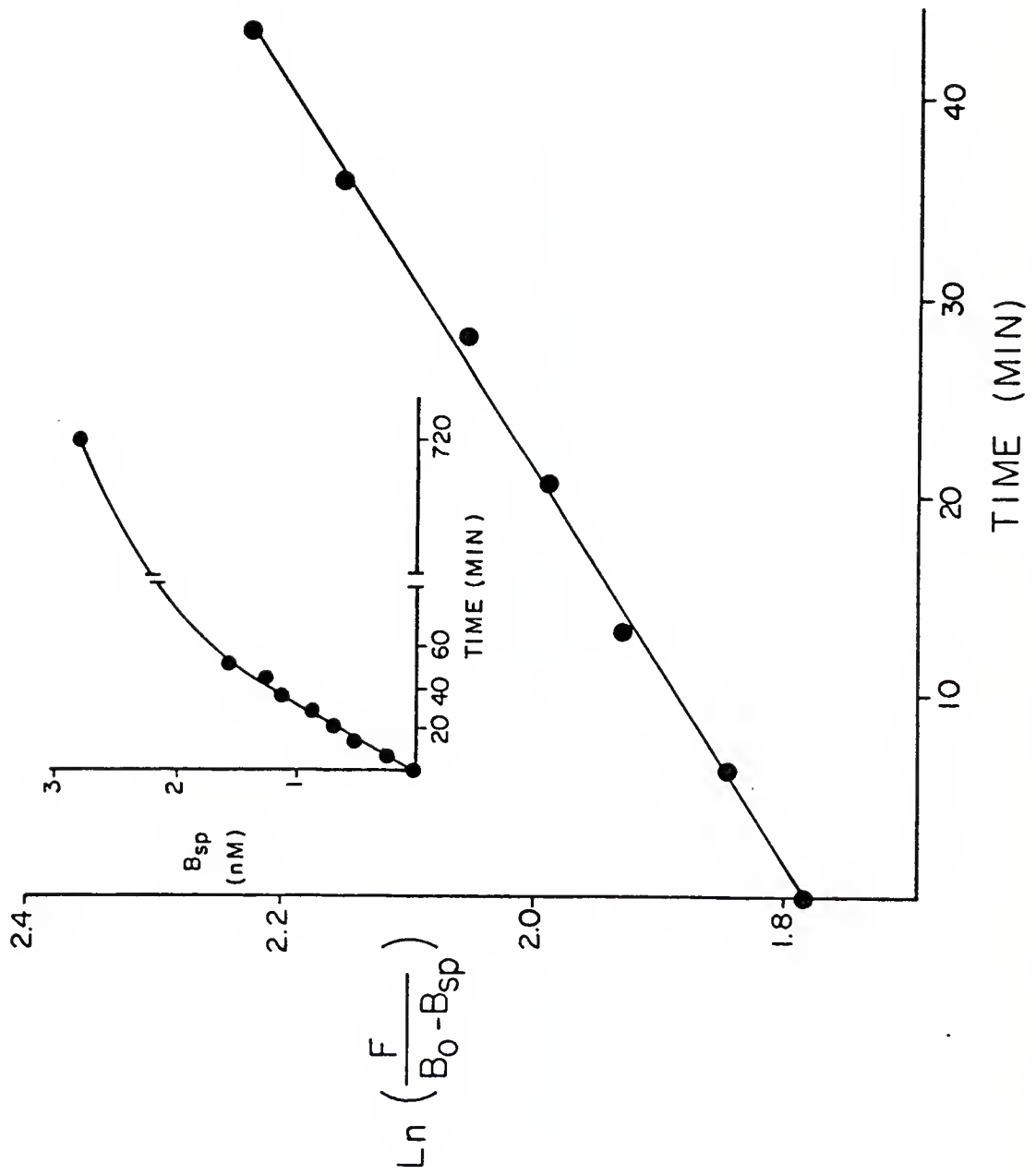

Ln (F / (B0 - Bsp))
2.4
2.2
2.0
1.8
10
20
30
40
TIME (MIN)
Bsp (nM)
3
2
1
20 40 60
720
TIME (MIN)

Figure 6-2. Representative experimental determination of the second-order rate constant describing the association of [^{3}H]TA with glucocorticoid receptors in perfused mouse brain cytosol at 0-2°C.

A pair of cytosol samples was prepared in buffer A, and at time zero (t=0) the samples were mixed with small volumes of buffer A containing sufficient [^{3}H]TA (and, for one sample of the pair, a 200-fold excess of unlabeled TA) to bring the final concentration of [^{3}H]TA to 17.4 nM. Triplicate 50 µl aliquots were removed from each sample of the pair as rapidly as possible (approximately every 7 min) for the determination of specifically-bound [^{3}H]TA (B_{sp}) by the DEAE filter assay. The receptor concentration (B_o) required for the calculation of the ordinate was derived (as described in chapter IV) from an equilibrium experiment performed with the same cytosol preparation (not shown; figure 4-11 is a representative example). The abscissa of the plot is the elapsed time from the initial mixing of [^{3}H]TA with the receptor preparation, and the ordinate is ln[(free ligand)/(free receptor)]. For short times the plot of this ordinate as a function of time is a line having slope $k_a(S_L - B_o)$, from which the association rate constant k_a may be derived immediately. The line was fit by least-squares linear regression. Association rate constant $k_a = 4.4 \times 10^5 M^{-1} min^{-1}$; receptor concentration B_o = 4.4 nM (392 fmole/mg cytosol protein); total [^{3}H]TA concentration S_L = 17.4 nM. The cytosol protein concentration was 11.3 mg/ml. Inset: data describing the appearance of specifically-bound [^{3}H]TA (B_{sp}) as a function of the time after mixing the cytosol with [^{3}H]TA. The data were transformed as described above and used, in conjunction with the estimate of receptor concentration (B_o), to calculate the association rate constant (k_a).

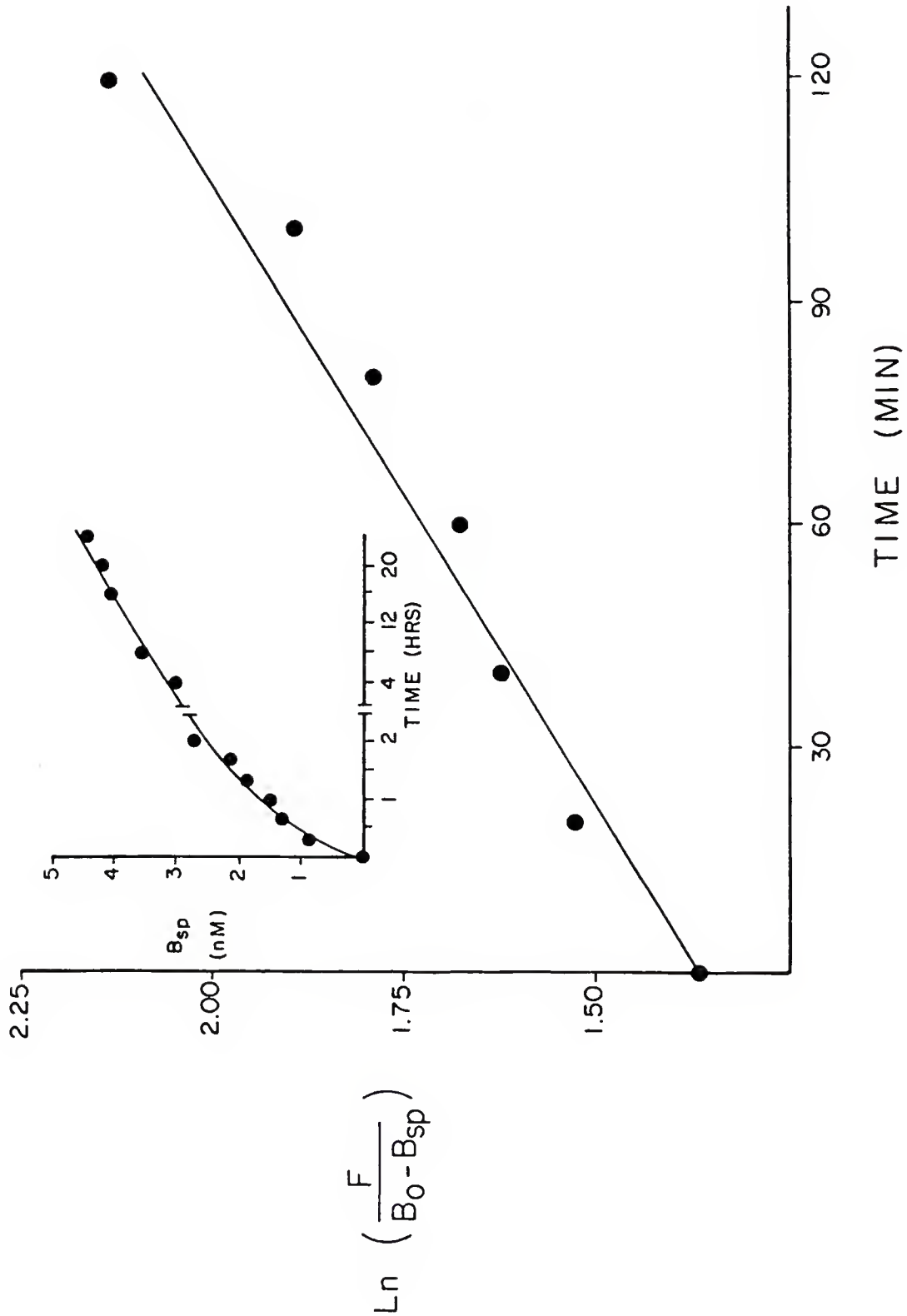

Ln (F / (B0 - Bsp))
2.25
2.00
1.75
1.50
30
60
90
120
TIME (MIN)
Bsp (nM)
5
4
3
2
1
1
2
4
12
20
TIME (HRS)

Figure 6-3. Representative experimental determination of the second-order rate constant describing the association of [^{3}H]corticosterone with glucocorticoid receptors in perfused mouse brain cytosol at 0-2°C.

A pair of cytosol samples was prepared in buffer A, and at time zero (t = 0) the samples were mixed with small volumes of buffer A containing sufficient [^{3}H]corticosterone (and, for one sample of the pair, a 200-fold excess of unlabeled dexamethasone) to bring the final concentration of [^{3}H]corticosterone to 27 nM. Triplicate 50 µl aliquots were removed from each sample of the pair as rapidly as possible (approximately every 7 min) for the determination of specifically-bound [^{3}H]corticosterone (B_{sp}, bound only to the putative glucocorticoid receptors and not to dexamethasone-resistant CBG-like binding sites) by the DEAE filter assay. The receptor concentration (B_o) required for the calculation of the ordinate was derived (as described in chapter IV) from an equilibrium experiment performed with the same cytosol preparation (not shown; figure 4-13 is a representative example). The abscissa of the plot is the time elapsed from the initial mixing of [^{3}H]corticosterone with the receptor preparation, and the ordinate is ln[(free ligand)/(free receptor)]. For short times the plot of this ordinate as a function of time is a line having slope k_a (S_L - B_o), from which the association rate constant k_a may be derived immediately. The line was fit by least-squares linear regression. Association rate constant k_a = 4.9 x $10^5 M^{-1} min^{-1}$; receptor concentration B_o = 1.8 nM (468 fmole/mg cytosol protein); total [^{3}H]corticosterone concentration S_L = 27 nM. The cytosol protein concentration was 3.8 mg/ml. Inset: data describing the appearance of specifically-bound [^{3}H]corticosterone (B_{sp}) as a function of the time after mixing the cytosol with [^{3}H]corticosterone. The data were transformed as described above and used, in conjunction with the estimate of receptor concentration (B_o), to calculate the association rate constant (k_a).

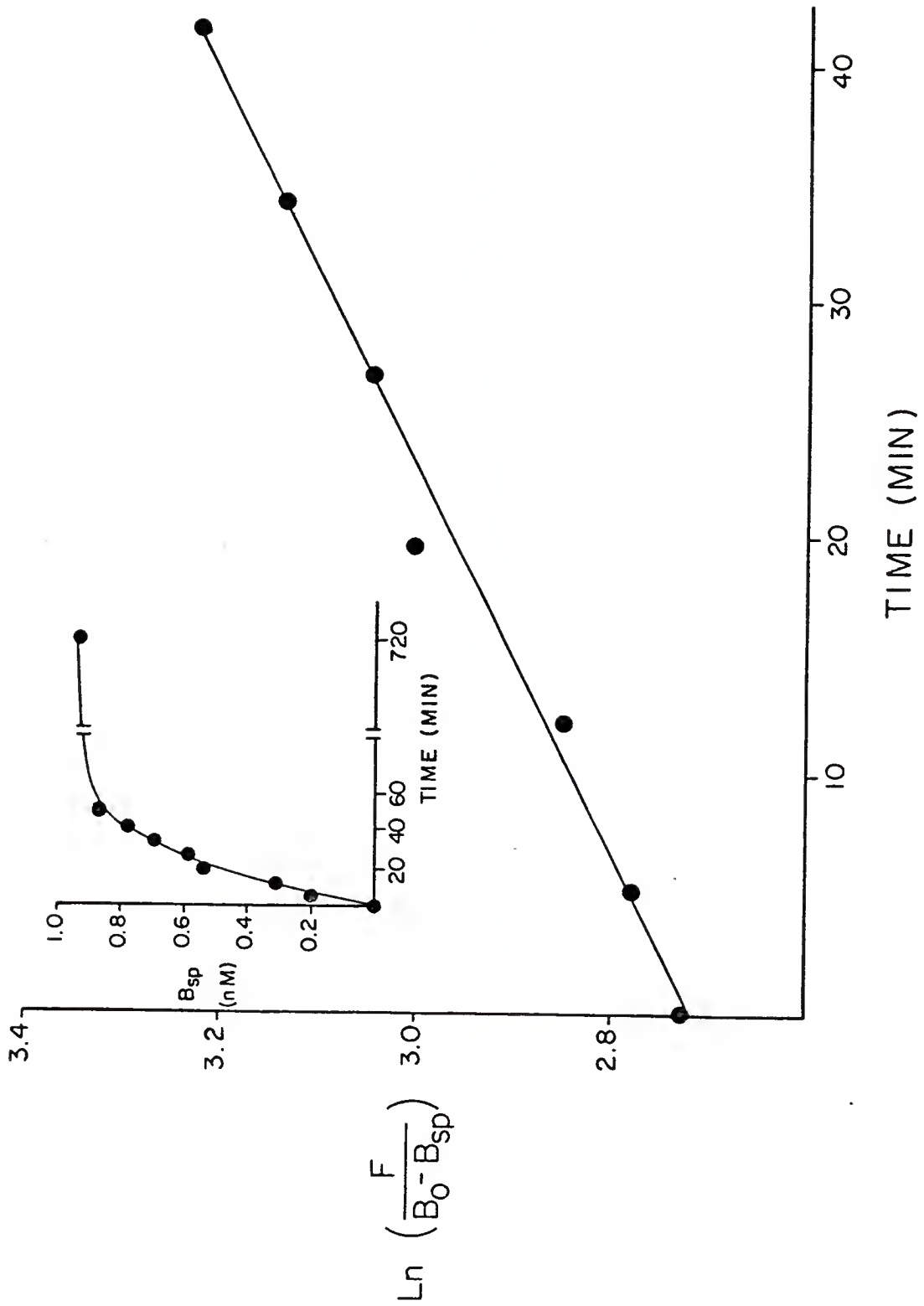

Ln (F / (B0 - Bsp))
3.4
3.2
3.0
2.8
10
20
30
40
TIME (MIN)
Bsp (nM)
1.0
0.8
0.6
0.4
0.2
20 40 60
720
TIME (MIN)

Table 6-1. Association and dissociation rate constants (and subsequently derived parameters) for the binding of [^{3}H]glucocorticoids to mouse brain cytosol receptors. The tabulated estimates are $\bar{x}$ ± s.e.m. Numbers within parentheses indicate either units of measure or the number of independent experiments that have been averaged. Steroid abbreviations are defined in the footnotes to table 4-2.

Parameter	[^{3}H]DEX	[^{3}H]TA	[^{3}H]B[a]
k_a ($\times 10^5 M^{-1} min^{-1}$)	6.9 ± 0.6(6)	4.4(1)	4.5 ± 0.5(3)
k_d[b] ($\times 10^{-4} min^{-1}$)	2.8 ± 0.2(3)	--	--
k_d[c] ($\times 10^{-4} min^{-1}$)	3.4 ± 0.2(3)	0.15(1)	49 ± 5(5)
k_d[d] ($\times 10^{-4} min^{-1}$)	7.7 ± 0.5(3)	--	--
$[k_d/k_a]$[c] ($\times 10^{-9} M$)	0.46 ± .07(3)	0.034(1)	9.5 ± 1.1(3)
$t_{\frac{1}{2}}$[c] (hrs)	34 ± 2(3)	780(1)	2.5 ± 0.2(5)
$t_{\frac{1}{2}}$[d] (hrs)	15 ± 1(3)	--	--

a. Binding to putative receptors only; nonspecific correction determined with 200-fold excess DEX.

b. Dissociation monitored during declining occupancy following treatment with dextran-charcoal; no additional unlabeled steroid added.

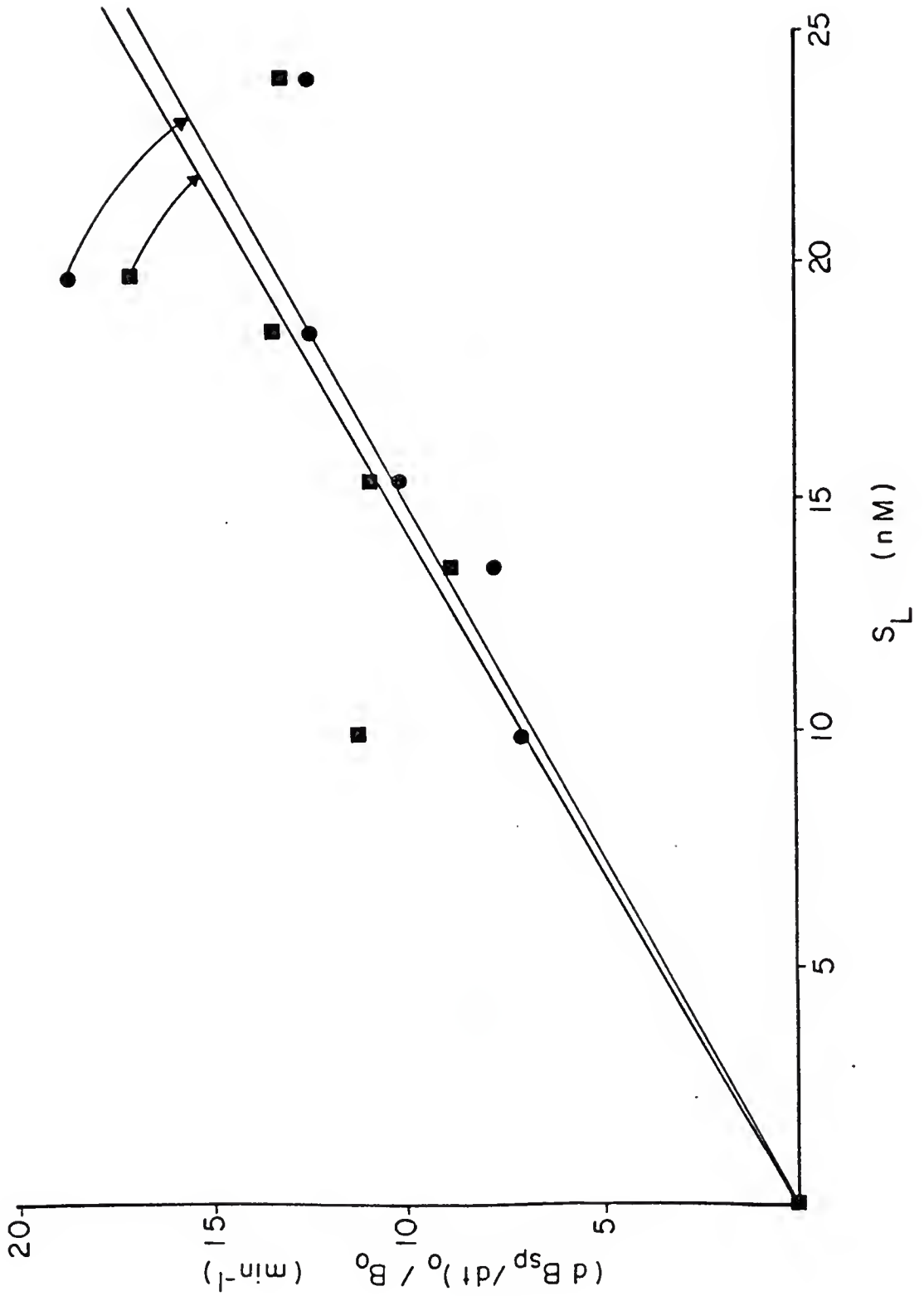
c. Dissociation monitored with receptors saturated in the presence of 25 μM unlabeled DEX.

d. Dissociation monitored in presence of 25 μM unlabled progesterone (P_4).

rate equation (6-1) reveals that the plot of dB_{sp}/dt (initial) as a function of S_L is a line possessing slope $B_o k_a$. In fact, the observed curvature of such a plot (a decrease in slope at high values of S_L) has even been offered as possible evidence that the glucocorticoid receptor binding process may involve multiple steps and unobserved transient intermediate states (Kaine et al., 1975; Pratt et al., 1975). (The most probable explanation of this anomaly is that in some experiments the constant incubation time was too long to provide a reliable estimate of the initial dB_{sp}/dt at the high ligand concentrations; e.g., Yeakley et al., 1980.) Aspects of the two different experimental designs that may be used for the determination of k_a have been combined in figure 6-4, in which the initial rate of increase of specific binding, dB_{sp}/dt (initial), has been plotted (after normalization by the receptor concentration B_o) as a function of total ligand concentration (S_L) for the six experimental determinations of the dexamethasone association rate constant (k_a). The ordinates were determined in two different ways: the initial slopes of the individual temporal binding curves (derived from the first three points of each curve) were taken as the estimates of dB_{sp}/dt (initial) to generate one set of points (■); the other set of points (●) was generated by calculating the initial slopes of the binding curves (i.e., $dB_{sp}/dt = k_a S_L B_o$ at t=0, from equation 6-1) from the values of k_a derived from the complete individual experiments as described above in "Methods". A line passing through the origin was fit to each set of points by least-squares regression. Although there was considerable scatter in the data, each set of points was at least consistent with the same straight line passing through the origin, indicating that the result of the inter-experiment "initial

Figure 6-4. Plot of the initial rate of increase of specifically-bound [^{3}H]dexamethasone (normalized by the receptor concentration B_o) as a function of the total ligand concentration S_L for the six experimental determinations of the [^{3}H]dexamethasone association rate constant (k_a).

The ordinates were determined in two different ways: first, the slope of the line fit by linear regression to the first three points of the temporal binding curve (e.g., inset to figure 6-1) was taken as the estimate of dB_{sp}/dt(initial) to generate one set of points (■); the other set of points (●) was generated by calculating the initial slope dB_{sp}/dt (i.e., equation 6-1: $dB_{sp}/dt = k_a S_L B_o$ at t = 0) from the value of k_a derived from the individual experiment as described in Methods (e.g., figure 6-1). A line was fit to each set of points by linear regression through a fixed point (the origin). Although there is considerable scatter in the data, each set of points is at least consistent with a straight line passing through the origin, indicating that the individual experiments generated independent measurements of k_a that were not correlated with the ligand concentrations (S_L); furthermore, the two lines were not different. The slope of the line gives an averaged or merged estimate for k_a: $k_a = 7.02 \times 10^5 M^{-1} min^{-1}$ (from the ■ points). Thus, the data do not support the existence of unobserved transient intermediate states or suggest that incubation times were too long in the experiments performed at the higher [^{3}H]dexamethasone concentrations.


20
15
10
5
(d Bsp/dt)o / Bo (min-1)
5
10
15
20
25
SL (nM)

slope" method was consistent with the independent measurements of k_a, which yielded linear plots of log [(free steroid)/(free receptor)] versus association time and which were not correlated with the total ligand concentrations employed (the correlation of k_a with S_L for the six individual dexamethasone experiments was -0.06). The slope of the line fit to the "initial slope" points (■) provided a "merged" estimate of $k_a = 7.0 \times 10^5 M^{-1} min^{-1}$. Thus, the data were consistent with a simple bi-molecular association reaction and did not support the existence of any transient intermediate states that could not be assayed directly. Relatively high [^{3}H]dexamethasone concentrations (S_L) were used in the association experiments in order to improve the estimates of ($S_L - B_o$) and ($S_L - B_{sp}$) that were required for the calculation of k_a; hindsight suggests that several experiments employing smaller values of S_L (< 10 nM) should have been included in the series of independent determinations in order to improve the analysis depicted in figure 6-4.

Steroid Dissociation Rate

Figures 6-5, 6-6, and 6-7 depict representative experimental determinations of the rate constants (k_d) for the dissociation of [^{3}H]dexamethasone, [^{3}H]TA, and [^{3}H]corticosterone, respectively, from glucocorticoid-receptor complexes in perfused mouse brain cytosol; summary results with standard errors for the dissociation rate constants measured with the three [^{3}H]ligands are presented in table 6-1. The dissociation rate constant for [^{3}H]dexamethasone was measured at high receptor occupancy, with receptor occupancy declining, and in the presence of several different high concentrations of progesterone; the

Figure 6-5. Determination of the rate constant (k_d) for the dissociation of [^{3}H]dexamethasone from mouse brain cytosol receptors at 0-2°C in buffer A, and the effect of progesterone on the dissociation rate constant.

A pair of cytosol samples was incubated at 2°C for 12 h with 6 x 10^{-8}M [^{3}H]dexamethasone and, for one of the samples, 200-fold excess unlabeled dexamethasone. Free dexamethasone was then removed from each sample by three sequential treatments with dextran-coated charcoal (DCC). Aliquots (1.5 ml) of the "total binding" sample (not containing cold dexamethasone) were diluted into 75 ml portions of buffer A containing either no additional steroid (●), 25 μM unlabeled dexamethasone (▲), 6.3 μM unlabeled progesterone (PROG, ■), or 25 μM unlabeled progesterone (□) for the measurement of dissociation. Another 1.5 ml aliquot of the "total binding" sample was diluted into a 75 ml portion of buffer A containing 6 x 10^{-8}M [^{3}H]dexamethasone, and a 1.5 ml aliquot of the "nonspecific" sample was diluted into a 75 ml portion of buffer A containing both 6 x 10^{-8}M [^{3}H]dexamethasone and 200-fold excess cold dexamethasone; these samples were used as a control to determine the (unchanging) concentration of functional receptors in the dissociating preparations (not shown). A 1.5 ml aliquot of the "nonspecific" sample was also diluted into a 75 ml volume of buffer A containing 25 μM unlabeled dexamethasone; this sample served as the nonspecific correction that was subtracted from the assayed values of total binding in order to measure $B_{sp}(t)$, the specifically-bound [^{3}H]dexamethasone remaining in the dissociating samples. Triplicate 2.5 ml samples were removed periodically from each flask for the determination, by additional dextran-charcoal (DCC) assays, of the remaining specifically-bound [^{3}H]dexamethasone. The resulting values of $B_{sp}(t)$ were then used to calculate, as described above, the dissociation constants (k_d) for the different conditions. The ordinate is the ratio of the remaining specific binding, B(t), to the initially-bound [^{3}H]dexamethasone, B(0). The data are described by equation (6-5). Inset: Transformation of the data to the linear form (equation 6-4) that possess slope k_d. The line was fit by least-squares linear regression. Dissociation rates were: k_d(vehicle) = 3.2 x 10^{-4}min^{-1}($t_{\frac{1}{2}}$ = 36 h); k_d(25 μM DEX) = 3.8 x 10^{-4}min^{-1}($t_{\frac{1}{2}}$ = 30 h); k_d(6.3 μM PROG) = 4.8 x 10^{-4}min^{-1}($t_{\frac{1}{2}}$ = 24 h); k_d(25 μM PROG) = 8.7 x 10^{-4}min^{-1}($t_{\frac{1}{2}}$ = 13 h). Receptor concentration before final dilution, B_o = 2.3 nM (295 fmole/mg protein); protein concentration before final dilution, 7.8 mg/ml; initial binding after final dilution, B(0) = 0.03 nM; residual [^{3}H]dexamethasone in dissociating flasks, 0.07 nM.

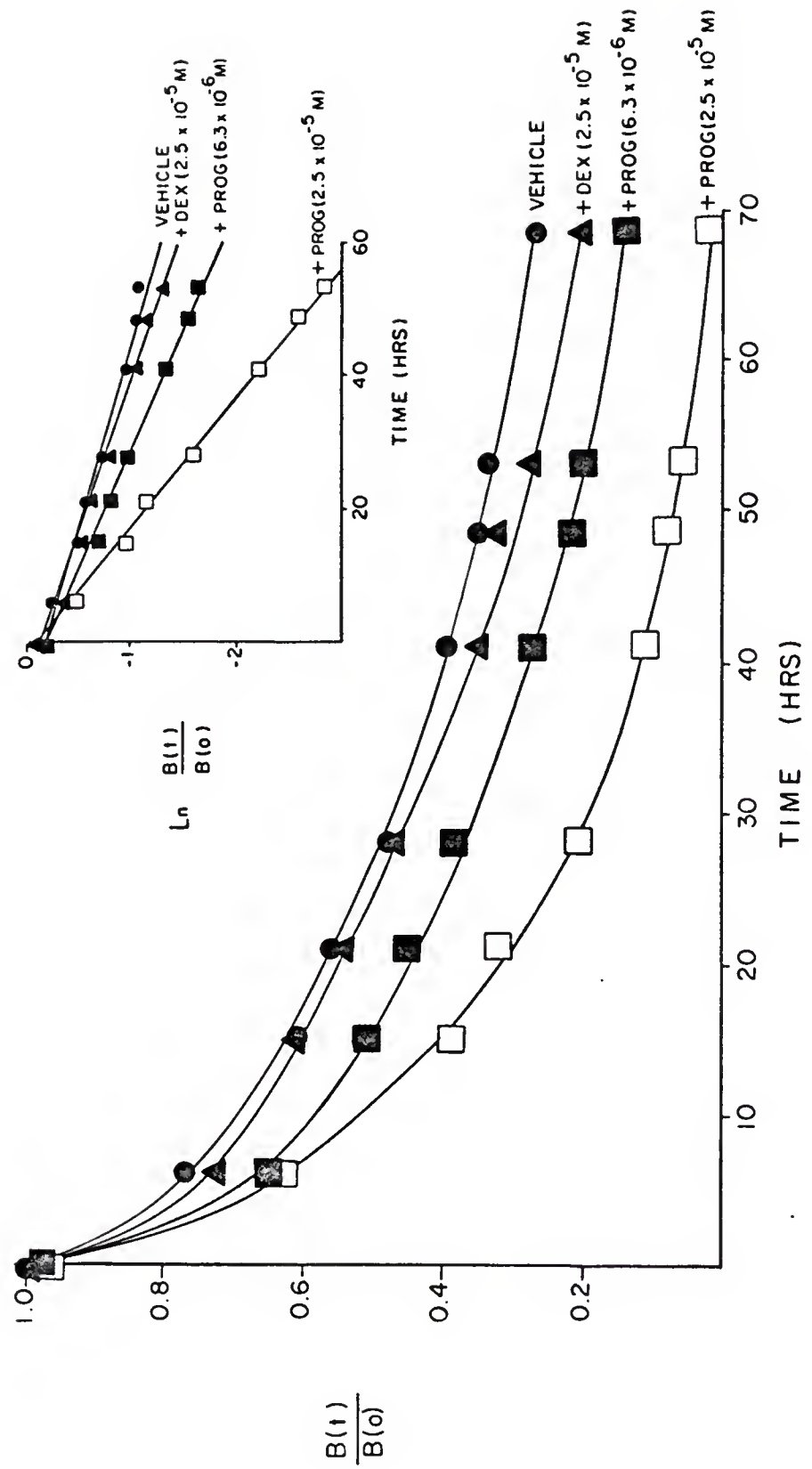

B(t)/B(o)
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
70
TIME (HRS)
VEHICLE
+ DEX (2.5 x 10⁻⁵ M)
+ PROG (6.3 x 10⁻⁶ M)
+ PROG (2.5 x 10⁻⁵ M)
Ln B(t)/B(o)
0
-1
-2
20
40
60
TIME (HRS)
VEHICLE
+ DEX (2.5 x 10⁻⁵ M)
+ PROG (6.3 x 10⁻⁶ M)
+ PROG (2.5 x 10⁻⁵ M)

Figure 6-6. Determination of the rate constant (k_d) for the dissociation of [^{3}H]TA from mouse brain cytosol receptors at 0-2°C for 12 h with 2 x 10^{-8}M [^{3}H]TA, and, for one of the samples, 200-fold excess unlabeled TA.

Free TA was then removed from each sample by three sequential treatments with dextran-coated charcoal (DCC). A 1.5 ml aliquot of the "total binding" sample (not containing cold TA) was diluted into a 75 ml portion of buffer A containing 25 µM unlabeled TA for the measurement of dissociation. Another 1.5 ml aliquot of the "total binding" sample was diluted into a 75 ml portion of buffer A containing 2 x 10^{-8}M [^{3}H]TA, and a 1.5 ml aliquot of the "nonspecific" sample was diluted into a 75 ml portion of buffer A containing both 2 x 10^{-8}M [^{3}H]TA and 200-fold excess cold TA; these samples were used as a control to determine the (unchanging) concentration of functional receptors in the dissociating preparation (not shown). A 1.5 ml aliquot of the "nonspecific" sample was also diluted into a 75 ml volume of buffer A containing 25 µM unlabeled TA; this sample served as the nonspecific correction that was subtracted from the assayed values of total binding in order to measure $B_{sp}(t)$, the specifically-bound [^{3}H]TA remaining in the dissociating sample. Triplicate 2.5 ml samples were removed periodically from each flask for the determination, by additional dextran-charcoal (DCC) assays, of the remaining specifically-bound [^{3}H]TA. The resulting values of $B_{sp}(t)$ were then used to calculate, as described above, the dissociation constant (k_d). The ordinate is the ratio of the remaining specific binding, B(t), to the initially-bound [^{3}H]TA, B(0). The data are described by equation (6-5). Inset: Transformation of the data to the linear form (equation 6-4) that possesses slope k_d. The line was fit by least-squares linear regression. Dissociation rate was: k_d(25 µM TA) = 1.5 x 10^{-5}min^{-1}($t_{\frac{1}{2}}$ = 780 h). Receptor concentration before final dilution, B_o = 4.4 nM (390 fmole/mg protein); protein concentration before final dilution, 11.3 mg/ml; initial binding after final dilution, B(0) = 0.05 nM.

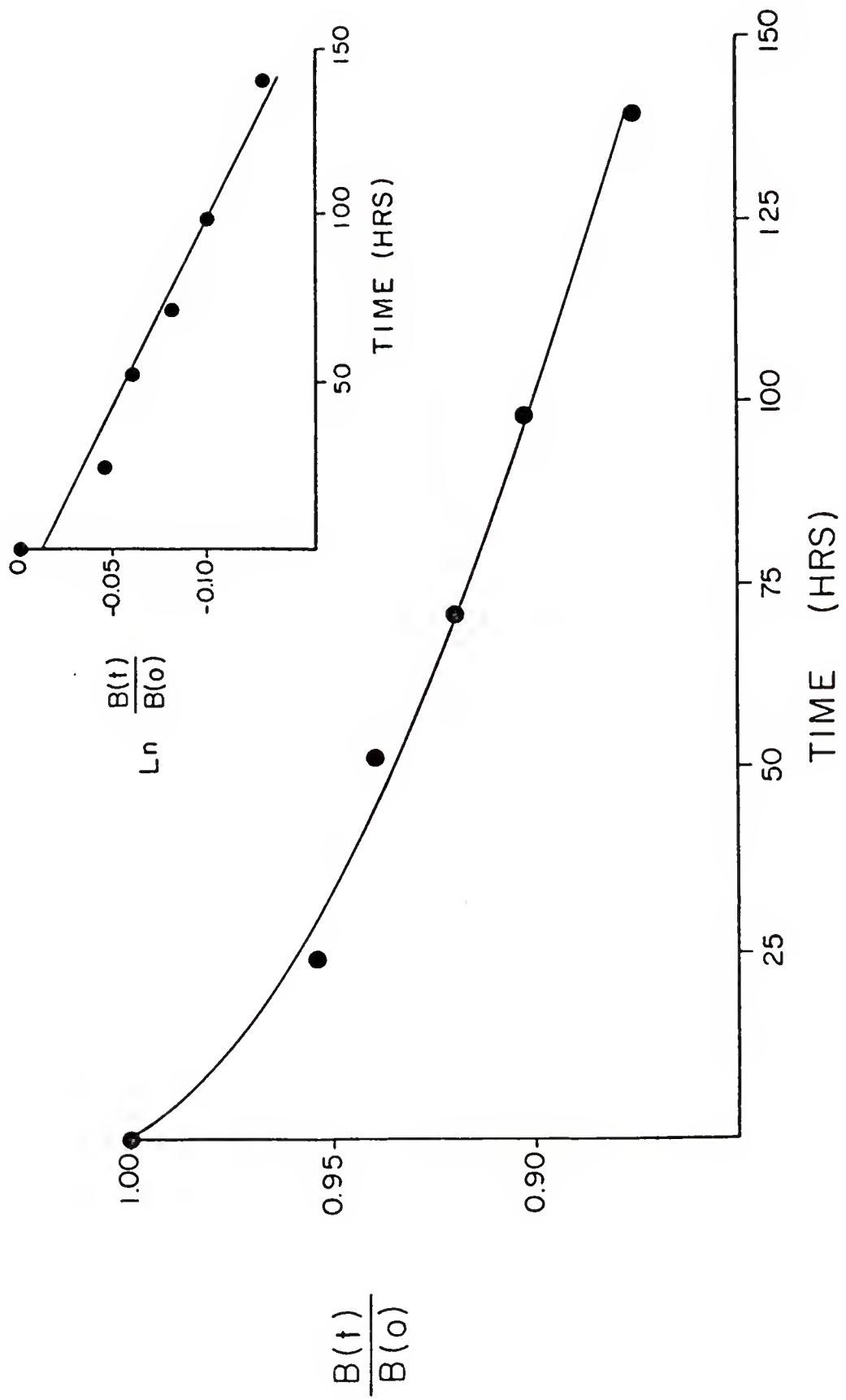
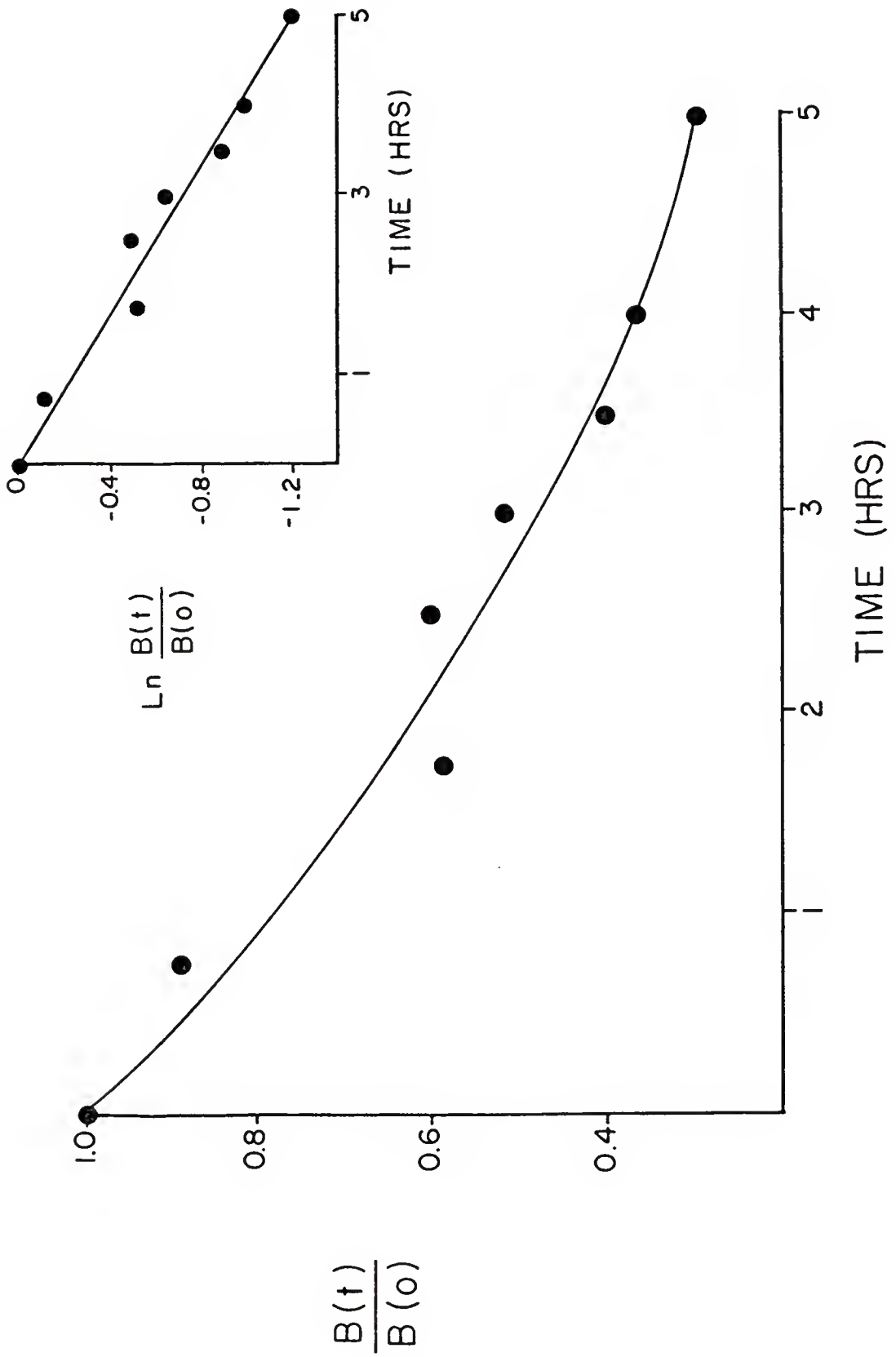

B(t)/B(0)
1.00
0.95
0.90
25
50
75
100
125
150
TIME (HRS)
Ln B(t)/B(0)
0
-0.05
-0.10
50
100
150
TIME (HRS)

Figure 6-7. Determination of the rate constant (k_d) for the dissociation of [^{3}H]corticosterone from mouse brain cytosol receptors at 0-2°C in buffer A.

A pair of cytosol samples was incubated at 2°C for 12 h with 6×10^{-8}M [^{3}H]corticosterone and, for one of the samples, 200-fold excess unlabeled dexamethasone to saturate the putative receptors but not the dexamethasone-resistant CBG-like binding sites. Following the incubation, an aliquot (1.125 ml) of the "total binding" sample (not containing cold dexamethasone) was mixed with buffer A (0.375 ml) containing sufficient cold dexamethasone to produce the concentration of 25 µM in the final dissociation sample (1.5 ml). Another aliquot (1.125 ml) of the "total binding" sample was mixed with buffer A alone (0.375 ml) to serve as the control in which the unchanging concentration of functional receptor sites was observed periodically (not shown). An aliquot (1.125 ml) of the "nonspecific" sample was mixed with buffer A (0.375 ml) containing cold dexamethasone to produce a concentration of 25 µM in the final 1.5 ml sample, which was used as the nonspecific background correction for both the dissociating sample and the control sample in which the concentration of functional receptor sites was monitored. Triplicate 50 µl samples were removed periodically from each of the three samples for the determination, by the DEAE filter asay, of the remaining specifically-bound [^{3}H]corticosterone in the dissociating sample. The resulting values of $B_{sp}(t)$ were used to calculate, as described above, the dissociation constant (k_d). The ordinate is the ratio of the specific binding, B(t), to the initial specific binding, B(0). The data are described by equation (6-5). Inset: transformation of the data to the linear form (equation 6-4) that possesses slope k_d. The line was fit by least-squares linear regression. Dissociation rate was: k_d(25 µM DEX) = $4.1 \times 10^{-3} min^{-1}$ ($t_{\frac{1}{2}}$ = 2.8 h). Receptor concentration, B_o = B(0) = 1.8 nM (470 fmoles/mg cytosol protein); cytosol protein concentration after dilutions, 3.75 mg/ml.


B(t)/B(o)
1.0
0.8
0.6
0.4
1
2
3
4
5
TIME (HRS)
Ln B(t)/B(o)
0
-0.4
-0.8
-1.2
1
3
5
TIME (HRS)

rate constants for [^{3}H]TA and [^{3}H]corticosterone were measured only at high occupancy, in the presence of high concentrations of the homologous unlabeled steroids. The dissociation rate constants measured at high occupancy (saturation) were 3.4 x 10^{-4} min^{-1} for dexamethasone, 0.15 x $10^{-4}min^{-1}$ for TA, and 49 x $10^{-4}min^{-1}$ for corticosterone. Although quite different from each other, these rate constants were within the range of the previously-reported constants describing the dissociation of these glucocorticoids from receptors in other tissues (e.g., Yeakley et al., 1980; Moguilewsky and Deraedt, 1981; Aranyi, 1982).

Figure 6-5 and table 6-1 reveal that high concentrations of progesterone accelerated the dissociation of [^{3}H]dexamethasone from the glucocorticoid receptors: the half-life ($t_{\frac{1}{2}}$) of [^{3}H]dexamethasone-receptor complexes was reduced from 34 h to 15 h by the presence of 25 μM progesterone. In a single experiment (not shown) a progesterone dose- response curve was generated by measuring the acceleration of the dissociation of [^{3}H]dexamethasone (as described above) in the presence of five different progesterone concentrations: 10^{-7}, $10^{-6.4}$, $10^{-5.8}$, $10^{-5.2}$, and $10^{-4.6}$ M(25 μM). The dose-response curve was analyzed by transformation to a "Scatchard-like"coordinate system (e.g., Svec et al., 1980) in which the abscissa was the concentration of progesterone [PROG], and the ordinate was Δk_d/[PROG], where Δk_d represents the difference between k_d measured in the presence of progesterone and the baseline value of k_d measured in the absence of progesterone (but with the receptors saturated with 25 μM dexamethasone). Because of the limited solubility of progesterone, the dose-response curve did not attain a plateau at a maximal acceleration of the glucocorticoid dissociation rate. Nevertheless, crude estimates of the "catalytic" K_m

for progesterone, the maximal acceleration of glucocorticoid dissociation Δk_d (max) produced by the progesterone, and a Hill coefficient "n" for the progesterone effect (exactly analogous to the Hill coefficient derived from an equilibrium binding isotherm) were derived from the Scatchard transformation of the data from the single experiment (not shown). These estimates were: K_m = 5 μM, Δk_d(max) = 6.2 x 10^{-4} min^{-1}, and Hill "n" = 0.9. Although a very large uncertainty is associated with these estimates, it was interesting to compare them with the reported characteristics of the acceleration by progesterone of glucocorticoid dissociation from AtT-20 pituitary tumor cell cytosol receptors (Svec et al., 1980). The investigation of AtT-20 cytosol receptors produced the following estimates: K_m(progesterone) = 78 μM, and Hill n = 1.5; the value of Δk_d(max) was reported incorrectly with inappropriate units and therefore cannot be compared easily to our estimate. Although our K_m for the allosteric acceleration of glucocorticoid dissociation from mouse brain receptors by progesterone is smaller than the estimate derived from the AtT-20 cytosol receptor studies, the large uncertainty associated with both estimates precludes any inference that the characteristics of the receptors from the two sources are different; both estimates are very high and obviously nonphysiological. The AtT-20 cytosol receptor studies generated a Hill coefficient n = 1.5 that suggested the possession of cooperative properties by the allosteric progestin binding sites on the glucocorticoid receptors (although nothing is known of the precise relationship between the occupancy of the allosteric sites and the magnitude of the acceleration of the dissociation rate); our estimated Hill coefficient

(n = 0.9) did not suggest any apparent cooperativity among the putative allosteric progestin binding sites.

Figure 6-5 and table 6-1 reveal that the apparent dissociation rate of [^{3}H]dexamethasone measured at declining occupancy in the absence of added unlabeled steroid was slightly slower than the rate measured at saturation in the presence of 25 μM dexamethasone (18% reduction). If excess unlabeled steroid is not added to the preparation at the beginning of the dissociation period, the possible reassociation of newly-dissociated labeled ligand or the association of the small amount of residual labeled steroid not removed initially may reduce the measured apparent dissociation constant; thus, the 18% reduction in the dissociation rate observed in the absence of added unlabeled steroid was probably an artifact of reassociation and should not be considered evidence for an allosteric effect of dexamethasone itself. The magnitude of this artifact was estimated approximately by using the complete solution to the rate equation to monitor the relaxation of the specific binding (B_{sp}) to the new, low-but-nonzero equilibrium value. First, the dissociation rate measured experimentally in the presence of 25 μM dexamethasone ($3.4 \times 10^{-4} min^{-1}$) was assumed for the variable k_d. The previously measured value of k_a($0.65 \times 10^6 M^{-1} min^{-1}$) was also assumed for the calculation. Estimated values of S_L(0.07 nM [^{3}H]dexamethasone), B_o (0.05 nM), and $B(0) = (B_L)_o$(0.03 nM) were also available. Now equation (2-12) was used with these values of the independent variables to calculate $B_{sp} = B_L$ for the same values of t at which measurements of B_{sp} were made during the dissociation. These calculated values of $B_L(t)$ were then used to derive, from the slope of a plot of t versus ln[calculated $B_L(t)/(B_L)_o$], the underestimated apparent dissociation rate constant

(k_d^1) that should have been observed in the presence of the measured concentration of residual [^{3}H]dexamethasone and in the absence of added unlabeled steroid. The calculated value of k_d^1 was $2.7 \times 10^{-4} min^{-1}$, which was even smaller than the rate actually observed in the absence of additional unlabeled steroid ($2.8 \times 10^{-4} min^{-1}$). Thus, the slightly different values of the dissociation rate constant k_d for [^{3}H]dexamethasone observed in the presence of 25 μM cold dexamethasone and in the absence of added unlabeled steroid were consistent with the absence of an allosteric effect of dexamethasone itself on the glucocorticoid dissociation rate and could be reconciled by considering the possibility of the reassociation of residual [^{3}H]dexamethasone. The value of k_d measured at high glucocorticoid occupancy (25 μM unlabeled dexamethasone) was taken as the dissociation rate constant used to derive the equilibrium dissociation constant K_d from the individual rate constants k_a and k_d.

Discussion

The ratios of the measured rate constants also provided estimates of the equilibrium dissociation constants for interactions of the three [^{3}H]ligands with the putative glucocorticoid receptors; these estimates of k_d were included in table 4-1 for comparison with the results of the equilibrium experiments. The equilibrium and kinetic estimates of k_d for [^{3}H]corticosterone were in reasonable agreement. In the case of each high-affinity synthetic ligand the measured equilibrium dissociation constant was larger than the ratio of the dissociation and association rate constants (k_d/k_a); this discrepancy was 8-fold for [^{3}H]TA and 4-fold for [^{3}H]dexamethasone. Although large, these discrepancies were

considerably smaller than those reported previously to arise from studies of the binding of glucocorticoids to cytosol preparations from other target tissues (e.g., Pratt et al., 1975; Yeakley et al., 1980). The reduction or elimination of the inactivation of unoccupied receptor sites in vitro achieved by the inclusion of molybdate and other protective buffer ingredients probably has contributed to the decrease in this discrepancy observed in our experiments. Although the discrepancies that we have observed are considerably smaller than many that have been reported, they remain significant for TA and dexamethasone. We cannot explain these discrepancies easily as artifacts resulting from the failure to reach equilibrium in the TA and dexamethasone isotherm experiments (see chapter IV), since no increase in specific binding in the tubes containing low ligand concentrations was observed when incubations were extended beyond the reported times (see chapter IV). Furthermore, the unoccupied sites were inactivated at a negligible rate in the standard buffer A, so that it is unlikely that an apparent equilibrium was merely a temporary steady state resulting from the combination of increasing receptor occupancy and a concomitant loss of unoccupied sites. Agreement of the equilibrium and kinetic estimates of K_d for the lower-affinity ligand corticosterone was reassuring, but an explanation for the residual discrepancies for dexamethasone and TA remains elusive.

In agreement with published rate constants describing the association of the three [^{3}H]ligands with glucocorticoid receptors in cytosols prepared from other tissues (e.g., Aranyi, 1982; Aranyi and Quiroga, 1980; Yeakley et al., 1980), the measured rate constants for the asociation of the three glucocorticoids with mouse brain cytosol receptors

were very similar (table 6-1); the large differences in steroid affinity must therefore derive from the substantial differences in the rates of dissociation of the three glucocorticoid agonists from glucocorticoid receptors derived from all tissues that have been examined (e.g., table 6-1; Yeakley et al., 1980; Aranyi and Quiroga, 1980).

Although little variation among the association rates of the optimal glucocorticoid agonists has been observed, investigations with antagonists have revealed large differences in steroid-receptor association rates (e.g., Aranyi, 1982). All glucocorticoid-receptor association rates that have been observed are far too slow to be diffusion-controlled (e.g., Koren and Hammes, 1976), and they probably reflect moderate activation energies for steroid association. Determination of the second-order rate constants was made possible only by the extreme slowness of glucocorticoid association; these measurements could not have been performed with radioactive ligands if the association rates had been several-fold larger. Steroid association rate constants, like dissociation rate constants, are not immutable; changes in free Ca^{++} concentration have been observed to modulate the rate of association of dexamethasone with hepatoma (HTC) cytosol glucocorticoid receptors (Rousseau, van Bohemen, Lareau and Degelaen, 1982) without affecting the dissociation rate, and a specific inhibitor present in rat placental cytosol has been found to reduce the rate of association of progesterone with partially-purified presumptive progestin cytosol receptors from this tissue (Ogle, 1981).

The dissociation of the three glucocorticoid ligands from the mouse brain receptor sites was described adequately in each case by a single exponential decay term, consistent with the presence of a single class

of noninteracting glucocorticoid receptors (the rate of dissociation of [^{3}H]corticosterone from CBG-like cytosol binding sites was not measured). If dissociation kinetics are biphasic or complex under conditions of constant occupancy or saturation, then the possibility of several classes of binding sites must be considered. When kinetics are complex under conditions of declining occupancy, then the possibility of cooperativity must be considered; the experiment must then be repeated at high or constant occupancy to see if the apparent cooperativity is really a reflection of heterogeneity. Observations of complex estrogen receptor dissociation kinetics have correctly been attributed to the presence of a mixture of activated and nonactivated estradiol-receptor complexes in the dissociating cytosol preparations (dissociation of the nonactivated steroid-receptor complexes was faster than the dissociation of activated complexes: Weichman and Notides, 1980). On the other hand, the apparently complex [^{3}H]dexamethasone dissociation kinetics that were observed in rat hippocampal and pituitary cytosol have been prematurely attributed to receptor heterogeneity (Meyer and McEwen, 1982); whereas in fact, the absence of molybdate probably allowed a significant (and, which is even worse, time-dependent) fraction of the [^{3}H]dexamethasone-receptor complexes to become activated to the nucleophilic form in these experiments (Meyer and McEwen, 1982). The inclusion of molybdate has allowed us to measure rate constants that are consistent with the presence of a single population of nonactivated receptors. Very recently we have begun to investigate (also in the presence of molybdate) the dissociation rate of receptors whose nucleophilic activation has been confirmed by a DNA-cellulose binding assay; preliminary data have suggested that dissociation of the nonactivated

steroid-receptor complexes is more rapid than dissociation of the activated complexes, and that progesterone accelerates the dissociation rates of both activated and nonactivated complexes (Luttge and Gray, unpublished). These data suggest that nucleophilic activation alters the properties of the steroid binding sites, as well as the characteristics of other domains of the receptor molecules.

We have not yet reproduced the conditions of the experiments that demonstrated an acceleration of glucocorticoid dissociation following the preincubation (before addition of the [^{3}H]glucocorticoid) of rat thymic cytosol with low, physiologically-relevant concentrations of progesterone or promegestone (R5020) (Moguilewsky and Deraedt, 1981). It will be interesting to incubate receptors simultaneously with unlabeled progestin and excess [^{3}H]glucocorticoid, and then to measure the glucocorticoid dissociation rate following removal of all unbound steroid (and the further addition of unlabeled glucocorticoid); a gradual reduction of the glucocorticoid dissociation rate may be observed as the progestin dissociates from the secondary, allosteric binding sites. The nitration of tyrosyl residues by tetranitromethane (TNM) has been observed to inactivate the steroid binding sites of progesterone binding globulin (PBG) (Kuhn and Raymoure, 1982); it will also be interesting to see if this treatment can block the acceleration by progesterone of the dissociation of [^{3}H]dexamethasone from glucocorticoid receptors. It may also prove possible to investigate directly the properties of the allosteric binding sites with the use of [^{3}H]progestins, but this has not yet been accomplished.

CHAPTER VII

GENERAL DISCUSSION

The experiments reported here have examined a number of physiochemical characteristics of soluble mouse brain glucocorticoid binding sites, in order to determine whether the glucocorticoid receptor system in mouse brain resembles closely that operative in other target tissues and species. Although a few published studies have reported the existence of glucocorticoid receptors in the mouse brain (e.g., Finch and Latham, 1974; Nelson et al., 1976; Angelucci et al., 1980), no basic characterization of the kinetic and equilibrium binding parameters or the steroid specificity of these receptors has previously been reported. Although a body of literature concerned with the properties of rat brain glucocorticoid receptors already exists (for review, see: Bohus et al., 1982), recent improvements in receptor methodology have made it possible to study the brain glucocorticoid receptor system under conditions that prevent receptor activation (nucleophilic transformation) and maximize in vitro receptor stability, permitting the relatively lengthy incubations and procedures required to generate equilibrium isotherms and to investigate the size and shape of the receptors.

We have used the labeled glucocorticoids [^{3}H]corticosterone, [^{3}H]dexamethasone and [^{3}H]triamcinolone acetonide (cyclic acetal). [^{3}H]Dexamethasone, [^{3}H]TA and [^{3}H]corticosterone were used to measure equilibrium and kinetic binding parameters, whereas only the

nearly-irreversible ligand [^{3}H]triamcinolone acetonide ([^{3}H]TA) was used for relatively lengthy sedimentation and chromatography procedures required to examine receptor size and shape. The experiments reported here have explored buffer components and employed a mixture that prevents the loss of unoccupied binding sites at 2°C; used a rapid and convenient binding assay; considered the possible consequences of failure to allow adequate incubation time when ligand concentrations are low; compared equilibrium binding parameters derived from the same pool of experimental data by several different methods of analysis; determined ligand specificity by applying mathematically correct procedures to the analysis of steroid competition data; observed that CBG-like molecules contribute significantly to the total pool of corticosterone binding sites, and examined binding site sizes and shapes to assess the stability and homogeneity of the in vitro receptor population.

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BIOGRAPHICAL SKETCH

Harry E. Gray, III, was born March 24, 1943, in Nashville, Tennessee, to Dr. Mary P.E. Gray and Harry E. Gray, Jr. He graduated from Battle Ground Academy, Franklin, Tennessee, in 1960. He received training in physics, mathematics and astronomy at the University of Pennsylvania, from which he graduated in 1965; as an undergraduate he participated in research in low energy nuclear physics (electron spectroscopy). He became a graduate student in the Department of Physics and Astronomy, Vanderbilt University, where he was engaged in applied mathematical research related to the trajectories of planetary exploration vehicles; he withdrew from Vanderbilt University without receiving a degree. He has never served in the Armed Forces. He was employed by the Philadelphia (Pa.) Dept. of Health as a radiation physicist, by Litton Industries as an electronics technician, and by the Philadelphia (Pa.) School Board as an assistant teacher in a special education program for emotionally disturbed children. He taught mathematics and physics at Croydon Hall Academy, Atlantic Highlands, N.J. He participated in research on the earliest events of bacterial photosynthesis as biophysics research technician at the Johnson Foundation of the University of Pennsylvania. He participated in research on the perceptual processing of graphic materials as research technician in the Department of Psychology, Brandeis University. As research technician at the Center for Visual Science of the University

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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